

Full-length article

Time-dependence of cardiomyocyte differentiation disturbed by peroxisome proliferator-activated receptor α inhibitor GW6471 in murine embryonic stem cells *in vitro*¹Ling DING, Xing-guang LIANG, Yi-jia LOU²*Institute of Pharmacology and Toxicology and Biochemical Pharmaceutics, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China***Key words**peroxisome proliferator-activated receptor α ; p38 MAPK; embryonic stem cells; cardiomyocytes; differentiation¹ Project supported by the National Natural Sciences Foundation of China (No 30171121, 30472112, 30672564), the Key Grant of the Chinese Ministry of Education (No 03088), and the International Joint Key Grant of Zhejiang Province (No 2003C24005).² Correspondence to Prof Yi-jia LOU.
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Abstract

Aim: To investigate the possible roles of peroxisome proliferator-activated receptor α (PPAR α) and the signal pathway regulating the transcription of PPAR α in the cardiomyocyte differentiation course of murine embryonic stem (ES) cells *in vitro*. **Methods:** The expression of PPAR α during cardiomyocyte differentiation was analyzed using both Western blotting and immunofluorescence. Cardiac specific genes and sarcomeric proteins were evaluated when embryoid bodies were challenged with PPAR α specific inhibitor GW6471 at different time courses. The phosphorylation of p38 mitogen-activated protein kinase (MAPK) was studied in the differentiation process, and its specific inhibitor SB203580 was employed to study the function of p38 MAPK on cardiac differentiation and the expression of PPAR α . **Results:** The expression of PPAR α was observed to be at a low level in undifferentiated ES cells and markedly induced with the appearance of beating clusters. The inhibition of PPAR α by its specific inhibitor GW6471 (1×10^{-5} mol/L) significantly prevented cardiomyocyte differentiation and resulted in the reduced expression of cardiac sarcomeric proteins (ie α -actinin, troponin-T) and specific genes (ie α -MHC, MLC2v) in a time-dependent manner. In the differentiation course, p-p38 MAPK was maintained at a high level from d 3 followed by a decrease from d 10. The inhibition of the p38 MAPK pathway by SB203580 between d 3 and d 7 efficiently prevented cardiomyocyte differentiation and resulted in the capture of the upregulation of PPAR α . **Conclusion:** Taken together, these results showed a close association between PPAR α and cardiomyocyte differentiation *in vitro*, and p38 MAPK was partly responsible for the regulation of PPAR α .

Introduction

Peroxisome proliferator-activated receptor α (PPAR α) is a member of the nuclear receptor superfamily of transcription factors and binds cognate response elements as an obligate heterodimer with the retinoid X receptor. PPAR α plays a key role in the transcriptional regulation of genes encoding mitochondrial fatty acid β -oxidation (FAO) enzymes during cardiac development and in response to physiological and pathophysiological stimuli, including fasting, cardiac hypertrophy, and cellular hypoxia^[1-4]. FAO enzymes, includ-

ing medium chain acyl co-enzyme A, dehydrogenase, and muscle carnitine palmitoyltransferase I (M-CPT I or CPT I β)^[5-7], are the principal sources of energy production in adult mammalian cardiomyocytes. PPAR α is rich in tissues that have high energy demand, such as heart, skeletal muscle, brown fat, kidney, liver, and brain^[8], demonstrating a close association between PPAR α and energy turnover.

However, little is known about how physiological or pathophysiological signals are transduced for the modulation of the transcription of PPAR α in cardiomyocytes. The p38 mitogen-activated protein kinase (MAPK) signaling

pathway could be activated by cellular stressors in the heart, including ischemia, hypoxia, and hypertrophic growth stimuli^[9]. As mentioned earlier, the expression of PPAR α is modulated to adapt to different demands in these processes. Therefore, we hypothesize that p38 MAPK may act as the upstream event that regulates the transcription of PPAR α .

PPAR α , together with PPAR δ and PPAR γ , form a subgroup within the nuclear receptor superfamily^[8,10]. PPAR isoforms have been demonstrated to be involved in the differentiation of several cell types, including nerve cells, adipose cells, and some tumor cell types^[11–13]. Prominent increased mRNA of PPAR α has been observed in the heart during embryonic development starting on d 7 *in vivo*^[14,15]. This prominent expression abates before birth, suggesting that a period of PPAR α exposure may be critical to the normal development of the heart.

It is increasingly clear that the p38 MAPK pathway plays an important role in a large number of cellular processes, such as cell growth, cell differentiation, cell cycle arrest, and apoptosis^[9,16–20]. In mice, p38 MAPK activity was recently demonstrated to be required for the development of the 8–16 cell stage embryos^[17]. In the mouse embryonic carcinoma cell model, the activation of p38 MAPK has been shown to be necessary for cardiac differentiation^[16,21].

Although multiple roles for PPAR α have been proposed, little is known of the significance of PPAR α in early cardiac development, especially during the differentiation of cardiomyocytes. Using embryonic stem (ES) cells as a model system faithfully recapitulates cardiomyocyte differentiation^[22–24]. The present study was designated to determine the possible function of PPAR α in early cardiomyocyte differentiation of ES cells *in vitro*. In addition, the p38 MAPK pathway was also detected to evaluate the possible pathway regulating the transcription of PPAR α .

Materials and methods

Cell culture and differentiation The permanent ES cell line D₃ (American Type Culture Collection, CRL-1934, Manassas, VA, USA) was cultivated in undifferentiated states on primary cultures of mouse embryonic fibroblasts in Dulbecco's modified Eagle's minimal essential medium (DMEM, Gibco BRL, Life Technologies, Germany), supplemented with 10% fetal calf serum (FCS, Gibco, Germany), 1×10^{-4} mol/L beta-mercaptoethanol (Sigma, St Louis, MO, USA), non-essential amino acids (NEAA, Hyclone, Logan, UT, USA, stock solution dilution 1:100), and 1×10^6 units/L recombinant mouse leukemia inhibitory factor (Chemicon, Temecula, California, USA). For the differentiation of ES

cells, embryoid bodies (EB) were generated using the hanging drop method^[25,26]. On d 0, 30 microlitres of drops containing approximately 600 ES cells were placed on the lids of Petri dishes filled with D-Hanks' solution, and cultivated in hanging drops for 3 d followed by another 2 d in the Petri dishes. On d 5, the EB were plated separately onto gelatin-coated 24-well culture plates in differentiation medium that consisted of DMEM, 20% FCS, 1 μ mol/L mercaptoethanol, and 1% NEAA. GW6471 and SB203580 were added, respectively, at the time points indicated in the text.

Reagents SB203580 was purchased from Biomol (Plymouth Meeting, PA, USA). GW6471 was obtained from Sigma-Aldrich (USA). Each inhibitor was dissolved in DMSO at 1000 \times immediately prior to use. Unused inhibitor was aliquoted into Eppendorf tubes and stored at -20 $^{\circ}$ C.

RT-PCR The total RNA was isolated from the ES cells and EB using Trizol reagent (Gibco BRL, Germany) in accordance with the manufacturer's instructions. To synthesize first strand cDNA, 1 μ g total RNA was incubated with 0.5 μ g of oligo (dT) 6 primer (Sangon, Shanghai, China) and 5 μ L deionized water at 65 $^{\circ}$ C for 15 min. Reverse transcription reactions of 20 μ L were performed with 200 units of M-MuLV reverse transcriptase (Gibco BRL, Germany), 4 μ L of 5 \times reaction buffer, and 1 mmol/L deoxynucleoside triphosphate (dNTP) mixture for 1 h at 42 $^{\circ}$ C. Polymerase chain reactions of 50 μ L contained 1 μ L of the RT reaction product, 5 μ L of 10 \times PCR buffer, 25 units Taq polymerase (Sangon, China), 1 μ L of 10 mmol/L dNTP mixture, and 30 pmol of each primer.

Primers, annealing temperature, product size, and the number of PCR cycles are depicted in Table 1. Products were analyzed in 1.5% agarose, ethidium bromide staining gels. β -actin was used as an internal standard. No PCR products were found without RT reaction and in water controls. RNA from 3 independent experiments were analyzed.

Western blot analysis The cells were washed with phosphate-buffered saline PBS, collected in radioimmuno-precipitation assay RIPA buffer (containing 0.2% Triton X-100, 5 mmol/L EDTA, 1 mmol/L phenylmethanesulfonyl fluoride PMSF, 0.01 g/L leupeptin, and 0.01 g/L aprotinin) and lysed for 30 min on ice. Aliquots were assayed for protein concentration using the Bio-Rad protein assay kit (Hercules, CA, USA); equal amounts of proteins were loaded per well on a 12% SDS-PAGE. Subsequently, the proteins were transferred onto 0.45 μ m pore size nitrocellulose membranes and blocked with 5% dry milk in PBS (pH 7.4, with 0.1% Tween 20) at room temperature. The blots were probed with either rabbit polyclonal anti-PPAR α , mouse monoclonal anti-troponin-T, goat polyclonal anti-actin (dilution 1:500, Santa Cruz

Table 1. The primers of beta-actin, MLC2v, and alpha-MHC.

Name	Forward/reverse (5'→3')	Annealing/°C	Product/bp	Cycles
β-actin	TGACGGGGTCACCCACACTGTGCCCATCTA	58	660	25
	CTAGAAGCATTTGCGGTGGACGATGGAGGG			
MLC2v	TGTGGGTACCTGAGGCTGTGGTTTCAG	61	189	35
	GAAGGCTGACTATGTCCGGGAGATGC			
α-MHC	CTGCTGGAGAGGTTATTCTCG	65	301	35
	GGAAGAGTGAGCGGCGCATCAAGG			

Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti- α -actinin (dilution 1:500, Sigma-Aldrich, USA), rabbit polyclonal anti-phospho-p38 MAPK or rabbit polyclonal anti-p38 MAPK (dilution 1:1000, Cell Signaling Technology, USA) antibodies overnight at 4 °C, followed by washing 3 times with PBS-Tween (0.1% Tween 20) at room temperature and challenged with horseradish peroxidase HRP-conjugated goat anti-rabbit, rabbit anti-goat, or mouse anti-mouse antibodies (dilution 1:4000, Affinity Bioreagents, Golden, CO, USA), respectively. The proteins were visualized autoradiographically with an enhanced chemiluminescent substrate (Pierce, Rockford, IL, USA) and scanned using a bio-imaging analyzer (Bio-Rad, USA).

Immunofluorescence analysis Differentiated EB that had been grown on coverslips were fixed for 20 min in methanol at -20 °C, followed by permeabilization in 0.1% Tween 20 in PBS. After washing in PBS three times, the EB on the coverslips were transferred to PBS containing 10% goat serum for 30 min at room temperature. The EB were then placed into blocking buffer containing mouse monoclonal anti- α -actinin antibodies (dilution 1:100) and incubated overnight at 4 °C, followed by incubation in blocking buffer containing fluorescein isothiocyanate (FITC)-labeled mouse anti-mouse antibody (1:500, Sigma-Aldrich, USA). For double staining, rabbit polyclonal anti-PPAR α and mouse monoclonal anti-troponin-T were added together with the last dilution of 1:100, followed by incubation in blocking buffer containing FITC-labeled mouse anti-mouse (1:250, Sigma-Aldrich, USA) and Texa red-labeled goat anti-rabbit (1:250, Santa Cruz Biotechnology, USA) antibodies. For the morphometric analysis for the expression of sarcomeric proteins, all aspects of cell processing, immunostaining, and imaging were rigorously standardized. Digital images were obtained from randomly selected fields using 10 \times objective lens and analyzed by the image analysis software of the confocal setup (Leica TCS SP2, Bensheim, Germany). For fluorescence excitation, the 488 nm band of the argon ion laser of the

confocal setup was used. Emission was recorded using a longpass LP505-nm filter set (Leica, Germany). Data are expressed as mean \pm SD calculated as a percentage of the cells in the controls. At least 5 different fields were measured for each dish.

Statistical analysis Student's *t*-test and ANOVA were used to determine the statistical significance of differences between the values for the various experimental and control groups. *P*<0.05 was considered statistically significant.

Results

***In vitro* cardiomyocyte differentiation of ES cells** The study protocol of cardiac differentiation of murine ES cells *in vitro* are shown in Figure 1, indicating the time points for RT-PCR, Western blotting, and immunofluorescence. The

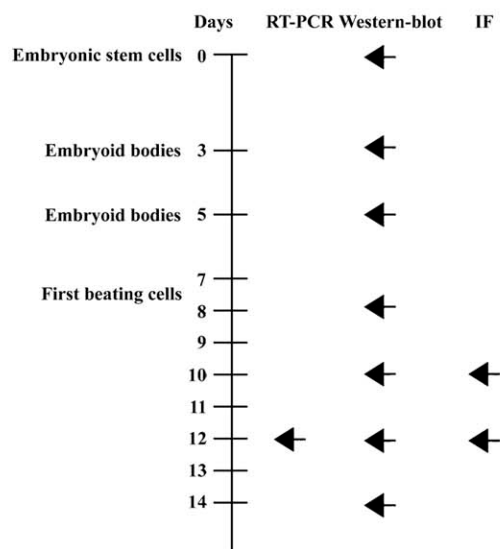


Figure 1. Schematic representation of murine ES cells cardiac differentiation protocol. Sample retrieval time points for RT-PCR, Western blotting, and immunofluorescence (IF) are indicated by arrows.

attached culture was established by plating a single, d 5 EB (Figure 2A) onto a 24-well plate and allowing continued cellular proliferation and differentiation. Within this multicellular arrangement in EB outgrowth, cardiomyocytes appeared as spontaneously contracting, round cell clusters. Each EB contained 1 or more beating areas (Figure 2B, 5 d after plating). An increase in size, strength of contraction, and beat frequency was observed during further differentiation. The synchronously contracting cardiomyocytes were positive for the anti-troponin-T antibody (Figure 2C). At higher magnification, cross striations were demonstrated with α -actinin immunolabeling (Figure 2E).

PPAR α increased during cardiomyocyte differentiation Differentiation of ES cells into cardiomyocytes first required their forming into EB by growing them in hanging drop cultures. After 3 d in hanging drops, the EB were placed

into suspension for 2 d, after which they were plated into individual wells of a 24-well plate. Beating commenced in distinct areas within EB on average 2 d later (culture d 7), the area of contracting cells expanded multifocally thereafter and reached a plateau around d 12. Therefore, these 2 time points were chosen for future analysis. As determined by Western blotting, the expression of PPAR α was at low levels in early differentiation, but increased obviously in parallel with the appearance of contracting cardiomyocytes (Figure 3A).

To confirm the result of the protein expression found by Western blotting, cultures were fixed at d 10 and doubly stained with antibodies against PPAR α and cardiac troponin-T. In each case, we selected fields which were full of cells so that a comparison could be made between the level of staining in the cells positive for the marker and those that were negative. There was a strong correlation between

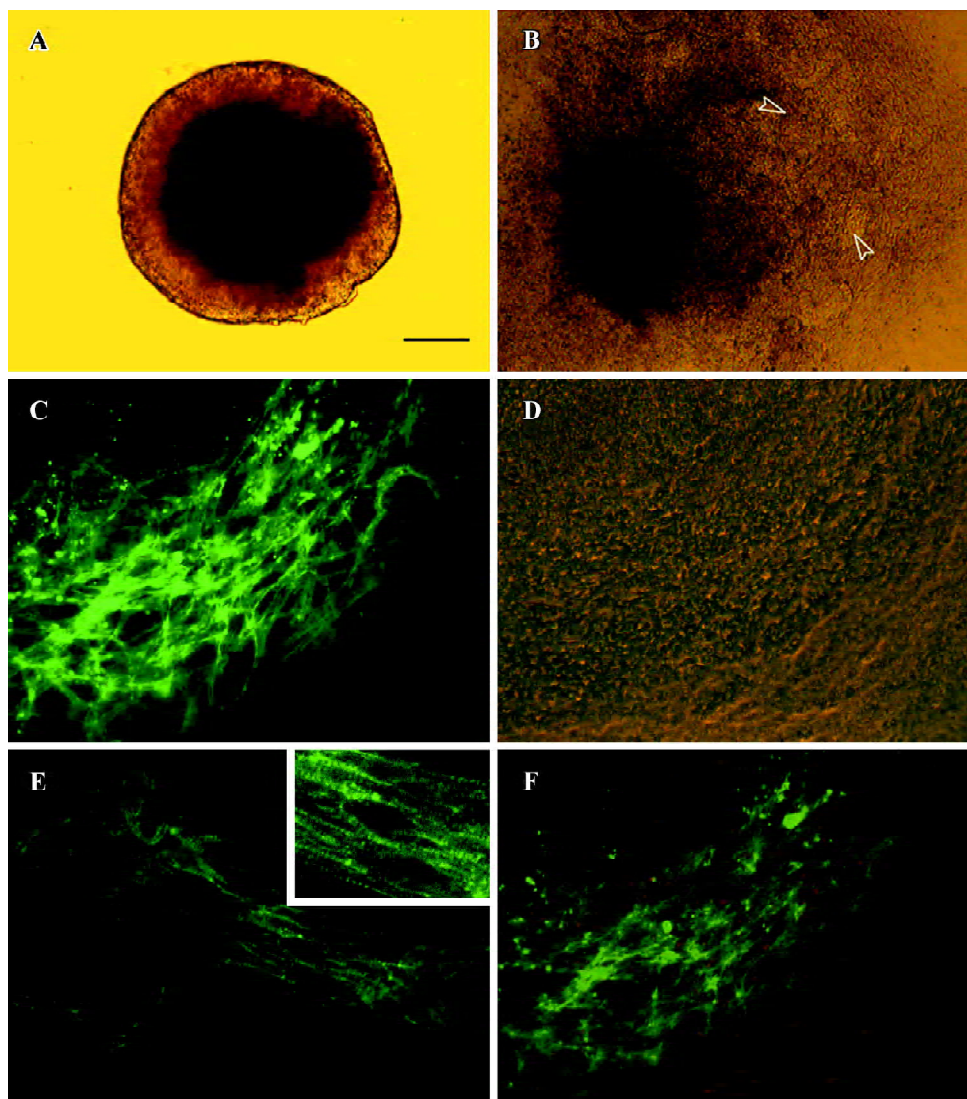


Figure 2. Cardiomyocyte differentiation of murine ES cells *in vitro*. (A) d 5 EB before plating onto gelatin-coated 24-well culture plates. (B) synchronously contracting, functional syncytium of cardiac clusters derived from EB (arrows: contraction foci). (C) EB were stained for anti-troponin-T antibody. C and D were taken from the same field. (E) EB were stained for anti- α -actinin antibody (insert: higher magnification reveals cross striations). (F) C and D were merged. Note that ES cells have differentiated into troponin-T positive cardiomyocytes. Scale bar=250 μ m (A, B), 125 μ m (C, D, F), and 40 μ m (E).

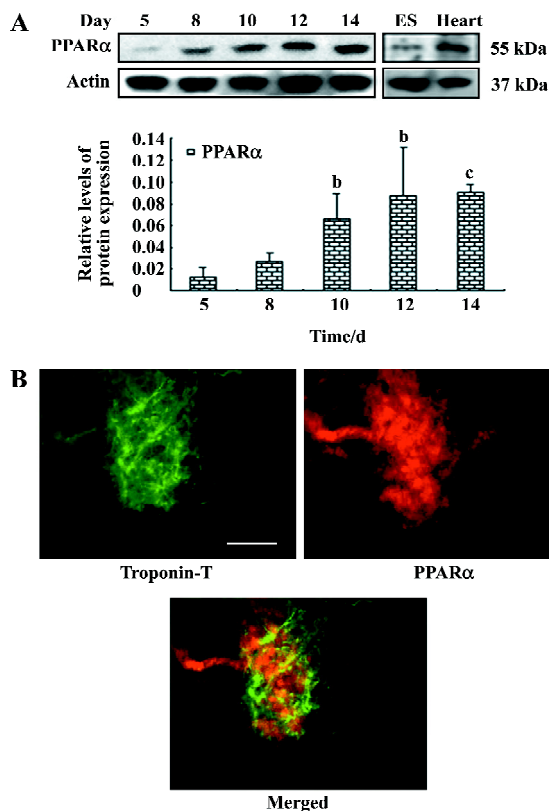


Figure 3. Expression of PPAR α during cardiac differentiation of ES cells *in vitro*. (A) PPAR α increased during cardiomyocyte differentiation of ES cells. On the days indicated, the samples were harvested and 80 μ g of each sample was run on 12% SDS-PAGE. Identical results were obtained for 3 independent differentiations. ^b $P < 0.05$, ^c $P < 0.01$ vs control. (B) EB at d 10 were doubly stained with antibodies against PPAR α and a differentiation marker cardiac troponin-T. The entire field of each figure was filled with cells. Scale bar=150 μ m.

PPAR α and troponin-T during differentiation; a high level of PPAR α was observed in cardiomyocytes positive for troponin-T (Figure 3B). Negative areas were also positive for PPAR α , but the staining was at considerably low levels.

Specific inhibition of PPAR α prevented cardiomyocyte differentiation of ES cells in a time-dependent manner GW6471, the specific inhibitor of PPAR α ^[27], was used in our experiment to address whether these changes in the PPAR α expression was a causative or a simple effect resulting from beating area formation. To closely define the exact time point during the course of the developmental program at which PPAR α activity was essential, GW6471 was applied at different time courses of differentiation. The data showed that only the application of GW6471 before d 9 could efficiently prevent EB from cardiomyocyte differentiation, indicated by the reduced formation of the beating area (Figure 4) and

expression of α -actinin and troponin-T (Figure 5B). When GW6471 was applied after d 9, cardiomyocyte differentiation and formation was slightly affected.

The expressions of α -MHC and MLC2v were analyzed by semiquantitative RT-PCR as they were specific transcription factors in cardiomyocyte differentiation. As a result, GW6471 tended to reduce the concentration of α -MHC and MLC2v compared with the appropriate controls (Figure 5A).

Inhibition of p38 MAPK prevented cardiomyocyte differentiation and reduced the expression of PPAR α To explore the function of p38 MAPK in cardiomyocyte differentiation and the expression of PPAR α , SB203580, the specific inhibitor of p38 MAPK, was employed in our experiment. P-p38 MAPK activity was maintained at a high level from d 3 and was followed by a decrease on d 10 (Figure 6A). The specific inhibition of p38 MAPK from d 3 to d 7 greatly reduced the formation of beating cardiomyocytes in the EB (Figure 6B) which was well matched with expression of PPAR α (Figure 6C). While treated with SB203580 from d 5, cardiac differentiation and PPAR α expression were just slightly affected. The expression of troponin-T was analyzed to confirm the inhibition of differentiation which was completely absent from the undifferentiated cells and the cells treated with SB203580 from d 3 to d 7. These results implied that the upregulation of PPAR α was restrained to cardiac differentiation of ES cells *in vitro*, and p38 MAPK might be the signal pathway regulating the expression of PPAR α .

Discussion

In this investigation, we gain insight into the expression and possible function of PPAR α during the cardiomyocyte differentiation of ES cells *in vitro*. PPAR α was observed to increase immediately after beating area formation. Interestingly, this phenomenon was recently replicated as the mRNA levels of PPAR α increased almost 10-fold during myogenesis^[28].

Cardiomyocyte differentiation can be divided into two processes: cardiogenesis and cardiac myofibrillogenesis. At d 3, cardiac transcription factors (ie GATA4, NKX2.5, MEF2C) are not expressed yet, while on d 5, cardiac transcription factors start to be fully expressed, but those encoding sarcomeric proteins are not. Since d 5 is a critical window at which the cardiac differentiation program becomes fully activated, but no cardiac cells identifiable by organized cardiac sarcomeric proteins (ie α -MHC, MLC2V) are yet present^[29], PPAR α appeared to mainly play a role in myofibrillogenesis. Our data showed that the inhibition of PPAR α before d 9 significantly disrupted differentiation and

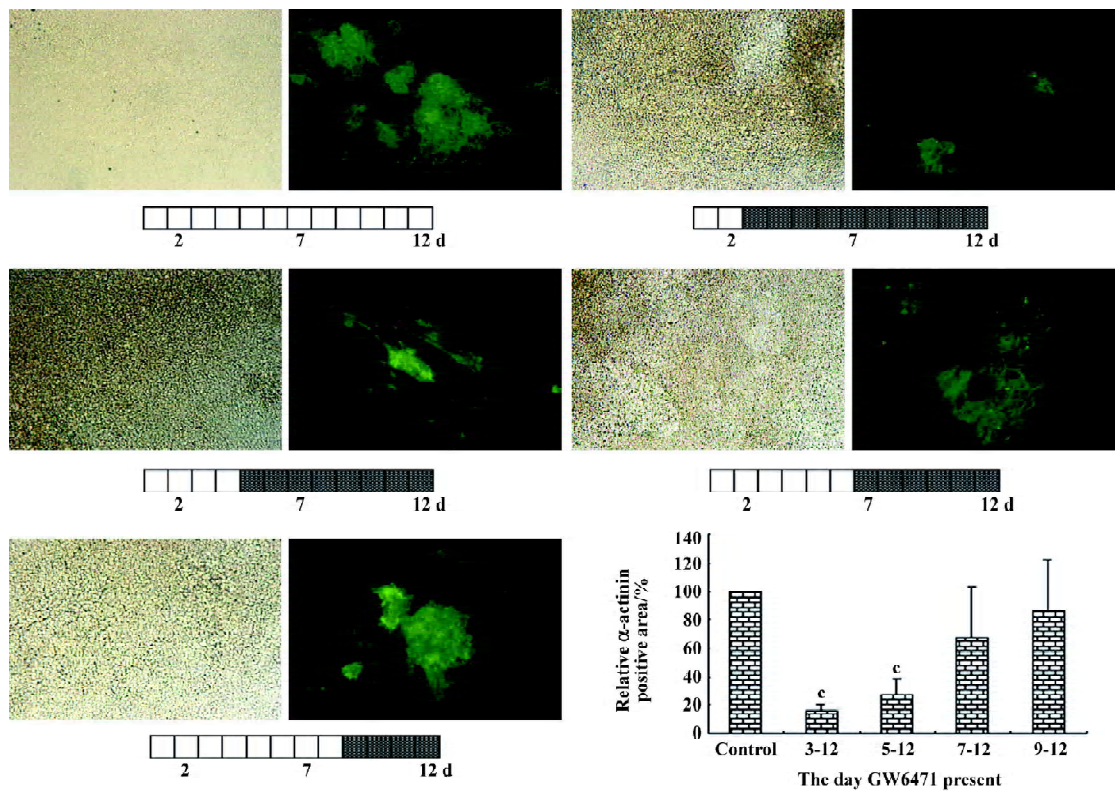


Figure 4. Time-dependence of cardiomyocyte differentiation disturbed by GW6471. EB were challenged with 1×10^{-5} mol/L GW6471 at different terms, and on d 12 of plating, the tissues were fixed and stained by the antibody against α -actinin. Fluorescence was recorded by means of confocal laser scanning setup and the area immunolabeled was evaluated by computer-assisted image analysis. Data are expressed as mean \pm SD calculated as a percentage of the cells in the controls. At least 5 different fields were measured for each dish. Scale bar=450 μ m. ^c $P < 0.01$ vs control.

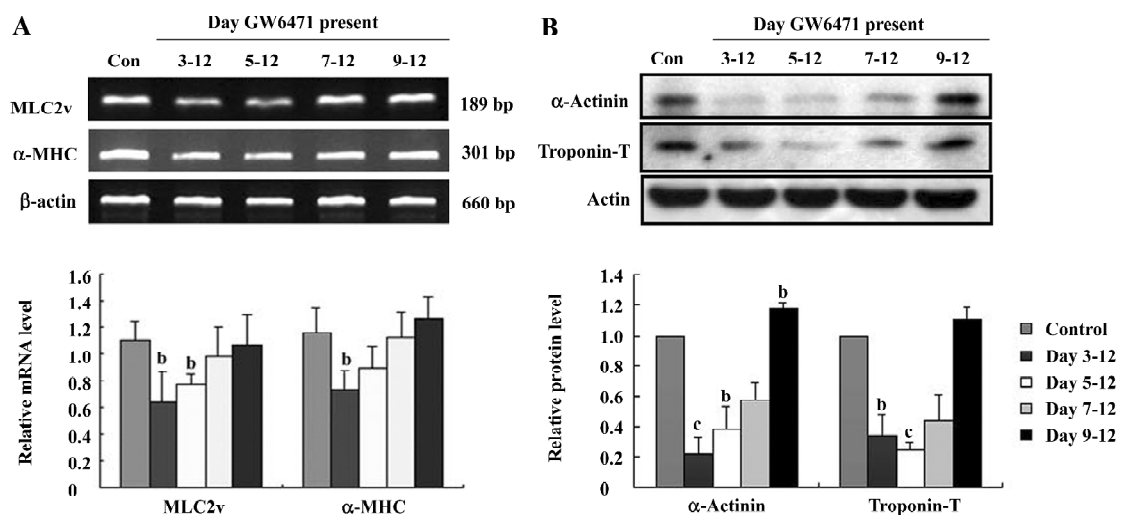


Figure 5. (A) EB were treated with GW6471 for different terms, and cardiac specific transcriptional factors were analyzed on d 12 by semiquantitative RT-PCR. cDNA was synthesized from 1 μ g mRNA and equivalent amounts of cDNA were amplified by PCR. β -actin gene was used for normalization. Each group contained 24 EB. (B) quantity analysis of cardiac troponin-T and α -actinin expression on d 12 when treated with GW6471 for different terms. Samples were harvested on d 12 and 100 μ g of each sample was ran on 12% SDS-PAGE. Each group contained 24 EB. The data are represented as mean \pm SD of 3 independent experiments. ^b $P < 0.05$, ^c $P < 0.01$ vs control.

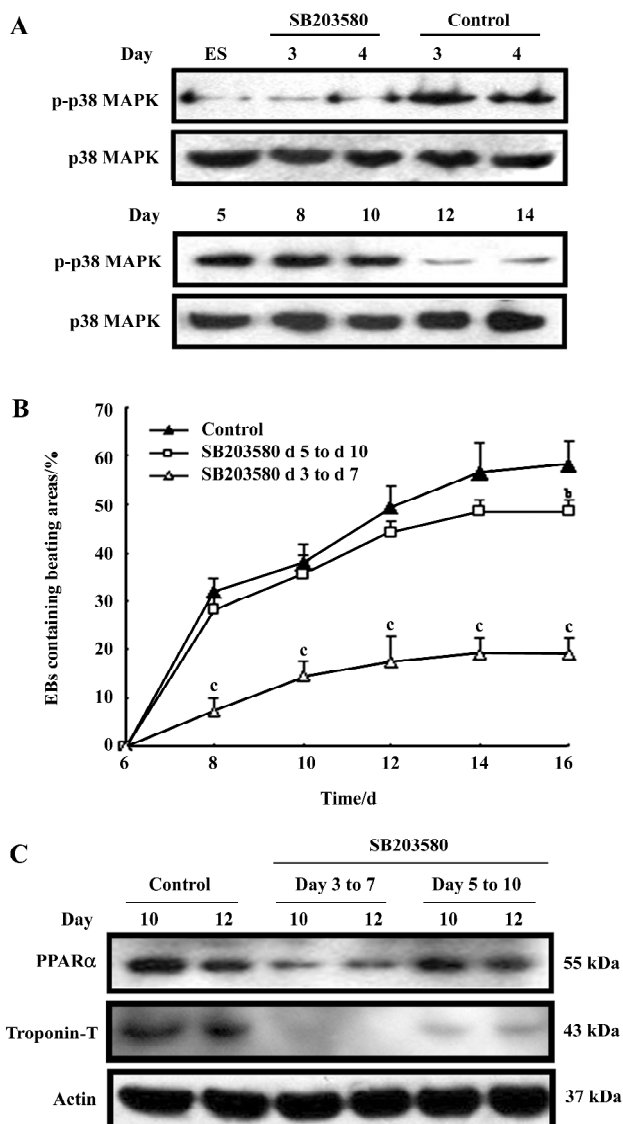


Figure 6. Function analysis of p38 MAPK on cardiomyocyte differentiation. (A) p-p38 MAPK was maintained at a high level from d 3 and decreased from d 10, and the application of SB203580 efficiently reduced the expression of p-p38 MAPK. (B) EB were treated with 1×10^{-5} mol/L SB203580 from d 3 to d 7 or from d 5 to d 12. $n=3$ independent experiments. Results are mean \pm SD. ^b $P < 0.05$, ^c $P < 0.01$ vs control. (▲) EB ($n=65$) treated with control medium; (□) EB ($n=70$) treated with SB203580 from d 5 to d 12; (△) EB ($n=66$) treated with SB203580 from d 3 to d 7. (C) increase of troponin-T and PPAR α were completely abolished when the cells were challenged with SB 203580 from d 3 to d 7. If SB203580 was presented from d 5 to d 10, the differentiation and expression of PPAR α could only be partially inhibited. Identical results were obtained for 2 independent differentiations.

reduced mRNA levels of α -MHC and MLC2v.

Mitochondrial number and functional capacity are

dynamically regulated in accordance with cardiac energy demands during developmental stages and in response to diverse physiological conditions^[30]. Since the cardiomyocyte differentiation of ES cells *in vitro* faithfully replicates the process *in vivo*, and these cardiomyocytes display properties similar to those observed in those *in vivo* or in primary cultures, there must be an increase in mitochondrial number with the emergence of beating clusters. In addition to the activation of suites of genes encoding contractile proteins, cardiac differentiation is accompanied by an organization of metabolism. Therefore, the most obvious explanation for our findings was that the inhibition of PPAR α impaired mitochondria ATP production or function of mitochondria, thus hampering normal development. That blockade of mitochondrial activity by the inhibition of mitochondrial protein synthesis, uncoupling of the inner membrane potential from ATP synthesis, and the inhibition of mitochondrial ATP production inhibits differentiation of skeletal muscle cells from myoblast precursors^[31]. The other explanation for our result may be that PPAR α was involved in the regulation of the nitric oxid (NO)/nitric oxid synthase (NOS) system and thus affected cardiac differentiation. The beneficial effects of the activation of PPAR α in the improvement of cardiovascular function have long been reported and these beneficial effects in cardiovascular diseases have been suggested to involve the NO/NOS system^[32,33]. Interestingly, NO signaling plays an important role in cardiac differentiation and NO treatment can accelerate mouse ES cells to differentiate into cardiomyocyte by both inducing a switch toward a cardiac phenotype and inducing apoptosis in cells not committed to cardiac differentiation^[34].

p38 MAPK was involved in cardiomyocyte differentiation of mouse embryonic carcinoma cells. Recently, p38 MAPK was further demonstrated to commit ES cells to cardiomyogenesis^[18]. Consistent with these previous studies, p-p38 MAPK was found to maintain at a high level from d 3 to d 10, which overlaps with the increase of PPAR α . Furthermore, the inhibition of p38 MAPK from d 3 to d 7 significantly prevented cardiac differentiation and reduced the expression PPAR α , which implied a relationship between PPAR α and p38 MAPK.

A previous study revealed that ligand-activated PPAR α would drive its own transcription^[35], and this effect would be increased if PPAR α was phosphorylated by p38 MAPK^[36]. There would have been abundant natural ligands (eg fatty acids) for the activation of PPAR α since EB were grown in the presence of 20% fetal bovine serum during differentiation. p38 MAPK-mediated phosphorylation activates PPAR α in a ligand-influenced manner and results in enhanced functional

cooperation with the transcriptional co-activator peroxisome proliferator-activated receptor γ coactivator (PGC)-1 α . Although we have no detailed experimental data on the mechanism by which p38 MAPK mediates the expression of PPAR α , two possibilities can be considered. First, p38 MAPK may enhance the binding between PPAR α and PGC-1 α and thus stimulates the transcription of PPAR α . Second, the activation of p38 MAPK can improve the stability of PPAR α during differentiation and thus contributes to the increase of PPAR α . However, we could not exclude the possibility that other upstream proteins might be responsible for the mediation of PPAR α during cardiac differentiation *in vitro*.

In conclusion, PPAR α increased in cardiomyocyte differentiation *in vitro*, and p38 MAPK partly acted as the upstream event regulating the expression of PPAR α . The inhibition of PPAR α in the early course markedly prevented cardiac differentiation, as indicated by the reduced expression of cardiac sarcomeric proteins and specific genes.

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