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Overexpression of *FABP3* Promotes Apoptosis Through Inducing Mitochondrial Impairment in Embryonic Cancer Cells

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ABSTRACT

Fatty acid-binding protein 3 (FABP3) is a low-molecular-weight protein with a distinct tissue distribution that may play an important role in fatty acid transport, cell growth, cellular signaling, and gene transcription. Previously, we have found that FABP3 was involved in apoptosis-associated congenital cardiac malformations, but the underlying mechanisms have not yet been described. In the present study, we investigated the characteristics of mitochondrial dysfunction in embryonic cancer cells (P19 cells) that overexpressed *FABP3*. We demonstrated that in *FABP3*-overexpressing P19 cells a lower cellular ATP production was accompanied by a dramatic decrease in mitochondrial membrane potential (MMP), despite the lack of a substantial decrease in the mtDNA copy number. In addition, *FABP3* overexpression also led to an imbalance in mitochondrial dynamics and to excess intracellular reactive oxygen species production. Collectively, our results indicated that overexpression of *FABP3* in P19 cells caused mitochondrion dysfunction that might be responsible for the development of FABP3-induced apoptosis. J. Cell. Biochem. 113: 3701–3708, 2012.

KEY WORDS: CONGENITAL HEART DISEASE; FABP3; MITOCHONDRION; P19 CELL

Congenital heart disease (CHD) is the leading non-infectious cause of death in children. Although the mortality of children with CHD has decreased because of early diagnosis and improved surgical treatments, not all CHD patients survive to adulthood. The cause of CHD is not yet fully understood, but it is becoming increasingly clear that many cardiac abnormalities that were once thought to have multifactorial etiologies are indeed attributable to defects in developmental control genes [Nemer, 2008; Bajolle et al., 2009].

Cardiac development is controlled by an evolutionarily conserved network of transcription factors that connect signaling pathways with genes for muscle growth, patterning, and contractility, which involves a wondrous and precisely orchestrated series of molecular and morphogenetic events. During evolution, this ancestral gene network has been expanded through gene duplication and co-option of additional networks. Mutations in components of the cardiac gene network or the subtle perturbation of this process can have catastrophic consequences in the form of CHD [Olson, 2006]. Due to the recent development of new molecular and developmental biologic techniques in the past decade, we have witnessed spectacular progress in elucidation of the molecular mechanisms of heart formation. In particular, several genes that include *NKX2.5* [Lints et al., 1993], *Tbx5* [Bruneau et al., 2001], and *GATA4* [Singh et al., 2010] have been identified as being essential for heart development.

Programmed cell death, or apoptosis, is an evolutionarily conserved process that plays a critical role in embryonic development and adult tissue homeostasis. In humans and mice, dysregulated apoptosis has been implicated in the pathogenesis of cancer and autoimmune, neurodegenerative, and cardiovascular

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diseases [Thompson, 1995]. Transgenic and knockout studies have both provided strong evidence for aberrant patterns of apoptosis that have resulted in CHD, including septation anomalies [Poelmann and Gittenberger-de, 2005]. Therefore, the preferential route of apoptosis has been postulated as an explanation for the observed differences in myocyte number/mass that accompanies the transition from the fetal to the adult circulatory system.

Fatty acid-binding protein 3 (FABP3) is a low-molecular-weight protein with distinct tissue distribution, which may play an important role in fatty acid transport, cell growth, cellular signaling, and gene transcription [Qian et al., 1999; Besnard et al., 2002]. FABP3 is upregulated during terminal differentiation of mouse cardiomyocytes [Tang et al., 2004]. In previous studies that used suppression subtractive hybridization, we identified genes that were differentially expressed between ventricular septum defect (VSD) myocardium and normal ventricular septum myocardium [Zhang et al., 2006].

In further studies, we also found that overexpression of *FABP3* can inhibits proliferation and promotes apoptosis and affects the differentiation of cardiac precursors into mature cardiomyocytes [Zhu et al., 2011]. This finding suggested that FABP3 may influence morphogenesis of the embryo heart by induction of increased myocardial cell apoptosis and be a promotive apoptotic gene. However, to date, the mechanism for the process has not been clarified. It is established that FABP3 participates in mitochondrion energy metabolism by transport of fatty acid during ATP production by the myocardial cell. Furthermore, the mitochondrion has been regarded more recently as a key element of apoptosis, as it plays a core role in the mammalian cell [Hoye et al., 2008]. Therefore, in the present study, we investigated the process of mitochondrion dysfunction, and whether mitochondrion impairment due to overexpression of *FABP3* was an important mechanism by which FABP3 promotes myocardial cell apoptosis, and the resulting delay in embryonic heart development. The P19 mouse teratocarcinoma cell line is known to be a suitable model for the study of cardiac differentiation at the molecular and functional levels [van der Heyden et al., 2003]. Therefore we selected the P19 cell line to investigate the effects of *FABP3* overexpression on mitochondrial function during the differentiation of P19 cells to cardiac myocytes.

MATERIALS AND METHODS

CELL CULTURE AND DIFFERENTIATION

The P19 cells used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were derived from a pluripotent tumor in an early mouse. The cells were cultured in α-modified Eagle's medium (α-MEM; Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2 in air. In order to induce cardiac differentiation, the cells were cultivated as aggregates from Day 0 to Day 4 in 15 ml α-MEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) on 10-cm bacterial dishes at 37°C in an atmosphere of 5% CO_2 in air. At Day 4, the embryoid bodies (EBs) were transferred to 6-cm tissue culture dishes, and cultured in α-MEM that contained 10% FBS for an additional 6 days. The morphological changes in the P19 cells were examined under an inverted microscope (Nikon, Japan) equipped with phase-contrast objectives and a digital camera (Nikon). In order to investigate how P19 cells can be differentiated into cardiac cells, we used Western blotting to identify the expression of cardiac troponin T (cTnT) using a monoclonal rabbit anti-cTnT antibody (CHEMICON, USA) during differentiation.

ESTABLISHMENT OF FABP3-OVEREXPRESSING CELLS

The coding sequence of mouse *FABP3* was amplified from P19 cells mRNA by reverse transcription polymerase chain reaction (RT-PCR) using a primer set (listed in Table I). The coding sequences were subcloned into the *Eco*RI and *Xho*I sites of a pcDNA3.1/*myc*–His B expression vector to generate a plasmid that expressed the *FABP3*–6x-His fusion protein. The resulting plasmids were confirmed by sequencing. The pcDNA3.1/*myc*–His B expression vector that carried the *FABP3* coding sequence or the vector alone was transfected into P19 cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. At 48 h after transfection, 600 μg/ml of neomycin (G418; Roche, Basel, Switzerland) was added to the medium to select transfected cells. Drug-resistant cells began to form small colonies at 2 weeks after the addition of G418. Individual colonies were then isolated and propagated. *FABP3*–6x His fusion protein was identified by Western blotting with an anti-6x-His antibody (Genetex, USA). Colonies that expressed the highest levels of FABP3 were selected for cell proliferation and differentiation studies.

MTT ASSAY

Stable overexpressing *FABP3* P19 cells and control cells (1×10^6/well) were seeded in 10-cm bacterial dishes and cultured in a-MEM supplemented with 10% FBS and 1% DMSO. At Day 4, the embryoid bodies were transferred to 96-well plates. Cell growth was monitored every 24 h for six consecutive using a Cell Proliferation MTT Kit (Roche Diagnostics, GmBH, Mannheim, Germany) as recommended by the manufacturer. The absorbance at 550 and 690 nm was recorded using an ELISA reader and the difference between these values was recorded as the OD.

APOPTOSIS ASSAY

Stable overexpressing *FABP3* P19 cells and control cells (1×10^6/well) were seeded in 10-cm bacterial dishes and cultured in a-MEM supplemented with 10% FBS and 1% DMSO. At Day 4, the embryoid bodies were transferred to 6-cm tissue culture dish. At Day 6–10, the

TABLE I. Oligonucleotide Sequences for Primer Sets

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Probe (5′–3′)
FABP3	AGCTGAATTCTGCAAT GGCGGACGCCTTTGTC	AGCTCTCGAGCGCG CCTCCTTCTCATAAG	– –
CYTB	TTTTATCTGCATCT GAGTTTAATCCTGT	CCACTTCATCTTA CCATTTATTATCGC	AGCAATCGTTCACC TCCTCTTCCTCCAC
28S	GGCGGCCAAGCGT TCATAG	AGGCGTTCAGTC ATAATCCCACAG	TGGTAGCTTCGC CCCATTGGCTCCT

cells were respectively harvested using trypsin/EDTA, washed with PBS, resuspended in 1 ml binding buffer, and stained with 10 μl annexin V-FITC and 10 μl PI at room temperature for 5 min (Biovision, CA, USA). The fluorescence of FITC and PI was analyzed using flow cytometry.

ELECTROMICROGRAPH MORPHOMETRY

Stable *FABP3*-overexpressing P19 cells or differentiated cells were collected after trypsin digestion and fixed in a mixture that contained 2.5% glutaraldehyde, 1.25% paraformaldehyde, and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4). The cells were then washed in 0.1 M cacodylate buffer, postfixed with 1% osmium tetroxide/1.5% potassium ferrocyanide for 1 h, washed in water, and stained in 1% aqueous uranyl acetate for 30 min, followed by dehydration in a gradient of different concentrations of alcohol (5 min in 70% alcohol, 5 min in 90% alcohol, and 5 min in 100% alcohol). The samples were then infiltrated and embedded in TAAB Epon (Marivac Canada Inc., St. Laurent, Canada). Ultrathin sections (about 60 nm) were cut on a Reichert Ultracut-Smicrotome, placed onto copper grids stained with uranyl acetate and lead citrate, and examined on a transmission electron microscope (JEOL JEM-1010, Tokyo, Japan) at an accelerating voltage of 80 kV.

REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) FOR MITOCHONDRIAL DNA (mtDNA)

Relative amounts of nuclear DNA and mtDNA were determined by real-time qPCR as described previously [Kaaman et al., 2007]. Briefly, DNA was isolated from P19 cells by using a DNA extraction kit (Qiagen, Germany). Two primer sets were used for PCR analysis. 110-nt mtDNA fragments within the cytochrome B gene (*CYTB*) gene were used for quantification of mtDNA. The PCR product had been cloned previously into the plasmid pMD-T18 and verified by DNA sequencing. Plasmid standards of known copy number were used to generate a log-linear standard curve, from which the *CYTB* copy numbers of the studied sample could be determined by qRT-PCR. Real-time qPCR was performed on an Applied Biosystems 7300 Sequence Detection System (ABI 7300 SDS; Foster City, CA). A 291-nt region of the 28S nuclear gene for was used as the reference gene to normalize the results. The plasmid standard curve that contained the 28S fragment was used to determine copy numbers of test samples. The ratio of mtDNA to nuclear DNA reflected the concentration of mitochondria per cell. The sequences of the primers and of the *Taq*man probe (Shenggong, Shanghai, China) are shown in Table I.

DETECTION OF ATP CONTENT

Adenosine triphosphate (ATP) was measured using an ATP Assay Kit (Beyotime, Nantong, China). Briefly, the transfected P19 cells were induced to differentiate as described above. On the 10th day of differentiation, the cells were homogenized in an ice-cold ATP-releasing buffer, and centrifuged at 12,000*g* for 5 min. After centrifuged, the supernatant was transferred to a new tube to test for ATP. The luminescence from a 20-μl sample was assayed in a single-tube luminometer (Turner Biosystems, CA) together with 100 μl of ATP detection buffer. The standard curve of ATP concentration (1 nM–1 μM) was prepared from a known amount of ATP. Luminescence was then normalized by protein concentration.

MEASUREMENT OF INTRACELLULAR OXIDATION LEVELS

Reactive oxygen species (ROS) generation was assessed using a 2′, 7′-dichlorodihydrofluorescein diacetate acetyl ester (H_2-DCFDA) probe (Sigma) as described previously [Maxwell et al., 1999]. After differentiation, cells were washed twice with phosphate-buffered saline (PBS) buffer and then incubated in pre-warmed α-MEM that contained 5 μM H_2-DCFDA at 37°C. After 30 min, the cells were washed three times with PBS. For flow cytometry, the cells were trypsinized and centrifuged at 300 rpm at 4°C for 5 min, and then resuspended in 300 μl PBS buffer, and then cells were analyzed with a FACScan flow cytometer using the CellQuest software (BD Biosciences, San Jose, CA; excitation at 488 nm, emission at 525 nm). The images were captured on a confocal laser scanning microscope. The fluorescence intensity represented intracellular ROS levels. In order to examine mitochondrial ROS, we isolated mitochondria using the Mitochondria Isolation Kit (Qiagen), and then incubated in pre-warmed α-MEM that contained 5 μM H_2-DCFDA at 37°C. The fluorescence was also analyzed with a FACScan flow cytometer.

MITOCHONDRIAL MEMBRANE POTENTIAL (MMP)

To prepare a stock solution, we dissolve the lyophilized MitoTracker® red probe (a red mitochondrion-specific cationic fluorescent dye that accumulates in mitochondria at a rate dependent upon the membrane potential; Molecular Probes, Invitrogen) in high-quality, anhydrous dimethylsulfoxide (DMSO) to a final concentration of 1 mM. The solution dyes was stored frozen at ≤−20°C and protected from light. Dilute 1 mM MitoTracker® stock solution to the final working concentration (150 nM) in appropriate FBS free medium. When cells have reached the desired confluency, remove the media from the dish and add prewarmed staining solution containing MitoTracker® probe for 30 min at 37°C, and then washed three times with pre-warmed PBS. For flow cytometry, the cells were trypsinized and centrifuged at 300 rpm at 4°C for 5 min, and then resuspended in 300 μl PBS buffer. Once inside the mitochondria, the dye cannot flow back outwards and is irrespective of the potential of the mitochondrial membrane. The spectral characteristics of the harvested cells were analyzed by fluorescence activated cell sorting (FACS; excitation at 579 nm, emission at 644 nm), and the images were captured on a confocal laser scanning microscope (Zeiss, Gottingen, Germany). The fluorescence intensity reflected the MMP.

STATISTICAL ANALYSIS

All data were expressed as means ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test or one-way analysis of variance (ANOVA) with the SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL). The threshold of statistical significance was defined as $P < 0.05$.

RESULTS

ESTABLISHMENT OF STABLE FABP3 OVEREXPRESSING CELLS AND APPRAISAL OF CELL DIFFERENTIATION

Stable *FABP3*-overexpressing P19 cells and the empty vector pcDNA3.1/*myc*–His B controls were established and maintained in α-MEM that contained 250 μg/ml of G418. Expression of FABP3 protein was verified by Western blot analysis (Fig. 1a). P19 embryonal carcinoma (EC) cells can differentiate into spontaneously beating cardiomyocytes in vitro [van der Heyden et al., 2003]. P19 cells were induced to differentiate towards cardiomyocytes by the addition of 1% DMSO. In order to investigate how P19 cells can be differentiated into cardiac cells, we used Western blotting to identify cTnT expression during differentiation. We found that cTnT expression was low before differentiation. The expression of cTnT was mainly upregulated after EB formation (Fig. 1b,c).

EFFECT OF FABP3 ON CELL VIABILITY

Cell growth was analyzed by MTT assay every day for a period of 6 days during differentiation. Overexpressing *FABP3* differentiated cells served as controls. As shown in Figure 2, *FABP3* overexpressing cells had a considerably lower proliferation rate compared with control cells during differentiation.

EFFECT OF FABP3 ON CELL APOPTOSIS

To study the effect of *FABP3* on differentiated P19 cells apoptosis, cells were cultured in a-MEM containing 0.9% DMSO for 5 days to induce differentiation. At different differentiated time points, cells were quantified by flow cytometric analysis after staining with annexin V-FITC. This showed that FABP3 promotes P19 cell apoptosis when the cells were induced differentiation ($^{*}P < 0.05$; Fig. 3).

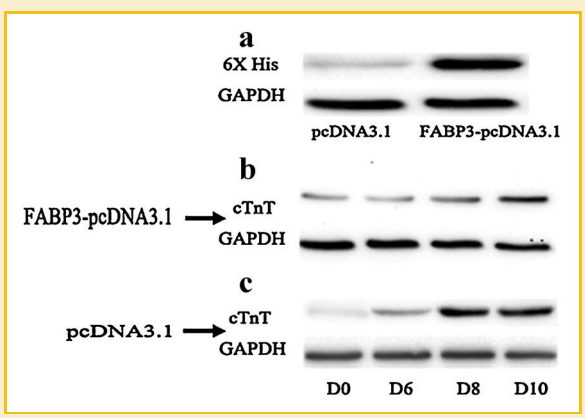

Fig. 1. Establishment of stable fatty acid-binding protein 3 (*FABP3*)-overexpressing cells and appraisal of cell differentiation. The pcDNA3.1*myc*/His B–FABP3 and pcDNA3.1*myc*/His B empty vectors were transfected stably into P19 cells, and total cellular protein was then extracted from the cells and subjected to Western blot analysis with a mouse anti-6x-His antibody (a). The cells were grown and treated with 1% dimethyl sulfoxide (DMSO; see Materials and Methods Section for details). Expression of the specific differentiation marker cTnT was determined by Western blotting at four time points during the differentiation process (Days 0, 6, 8, and 10; b,c). These data were based on three independent experiments, and similar results were obtained.

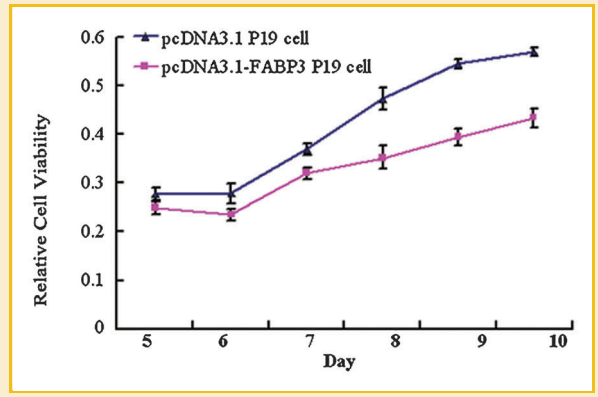

Fig. 2. Effect of fatty acid-binding protein 3 (FABP3) on cell viability. The effects of FABP3 on cell viability were detected by MTT assays. Data from the assay showed that FABP3 inhibit the proliferation of FABP3 during differentiation. Data resulted from three independent experiments and are expressed as the mean ± SEM of the difference between the absorbance at 550 nm ($^{*}P < 0.05$).

EFFECT OF FABP3 ON MITOCHONDRIAL MORPHOLOGY

We investigated the morphology of the mitochondria using electron microscopy. As shown in Figure 4, the mitochondria in *FABP3*-overexpressing cells showed abnormal morphology, such as condensed cristae, a smaller size and were twisted, with signs of mitochondrion damage that ranged from reduced density or swelling to virtually hollow mitochondria with reduced and missing cristae.

EFFECT OF FABP3 ON MITOCHONDRIAL DNA COPY NUMBER

The mtDNA copy number per mitochondrion is generally considered to be constant in most mammalian cell types [Robin and Wong, 1988]. The mtDNA copy number is regarded as the cellular mitochondrion number and could be a possible marker of

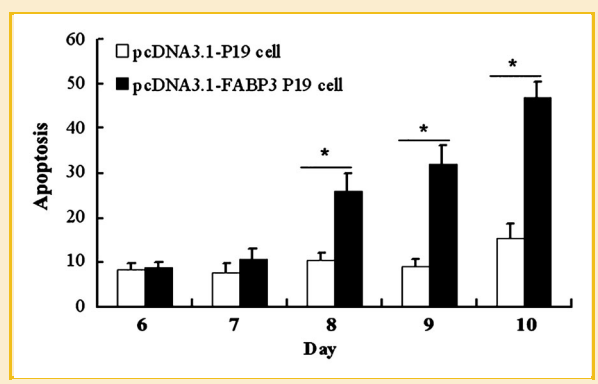

Fig. 3. Effect of fatty acid-binding protein 3 (FABP3) on cell apoptosis. Apoptosis was assayed by binding of annexin V-FITC. FABP3 was found to promote the apoptosis of P19 cells during differentiation ($^{*}P < 0.05$).

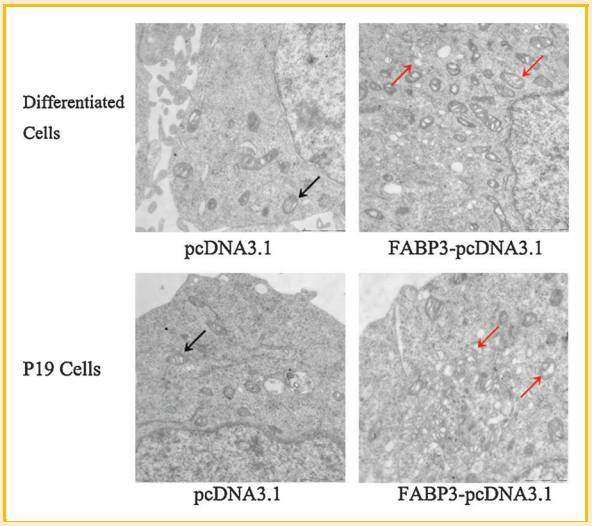

Fig. 4. Effect of fatty acid-binding protein 3 (FABP3) on the mitochondrion morphology in differentiated P19 cells. P19 cells transfected with pcDNA3.1-*myc*/His B–FABP3 or the empty vector (pcDNA3.1 *myc*/His B) were induced to differentiate, the ultrastructure of the mitochondria in the differentiated P19 cells was visualized using transmission electron microscopy. The scale bar in the bottom right band corner indicates 1 μm. The red arrows mean impaired mitochondria, and the black arrows mean normal mitochondria.

mitochondrion function. We measured mitochondrial and genomic DNA in *FABP3*-overexpressing and control cells using real-time qPCR. The results showed that there was no significant difference in mtDNA copy number between the two groups ($P > 0.05$; Fig. 5).

FABP3 DECREASED CELLULAR ATP PRODUCTION

To assess the effects of *FABP3* overexpression on mitochondrion function, we determined the total cellular ATP production in *FABP3*-overexpressing and control cells. Mitochondria are intracellular organelles that generate ATP through the process of oxidative phosphorylation [Brown, 1992]. As shown in Figure 6, *FABP3* overexpression resulted in a marked reduction in cellular ATP production. We also find that *FABP3* overexpression could decrease ATP production in undifferentiated P19 cells.

FABP3 INCREASED INTRACELLULAR ROS LEVELS

ROS, a byproduct of the electron transport chain, is mainly produced by mitochondria [Andreyev et al., 2005]. It has been proposed that increased ROS concentrations could lead to mitochondrion damage. We next sought to examine ROS levels in P19 cells. As shown in Figure 7, ROS levels in *FABP3*-overexpressing cells were markedly higher than that in the control cells, as indicated by more intense fluorescence signals in the presence of DFCDA. In undifferentiated cells, there was similar result.

FABP3 DECREASED MITOCHONDRIAL MEMBRANE POTENTIAL

To determine whether *FABP3* overexpression has an effect on mitochondria, we monitored MMP in differentiated P19 cells or undifferentiated cells after the stable transfection of *FABP3*. Whether differentiation or undifferentiated cells, *FABP3* overexpression resulted in a drop in MMP (Fig. 8), as shown by the membrane potential-dependent fluorescence probe. In the undifferentiated P19 cells, the result was the same.

DISCUSSION

In previous studies [Zhang et al., 2006], we have found that FABP3 is involved in apoptosis-associated congenital cardiac malformations, but the definitive mechanism has not been described. In the present study, we investigated the characteristics of mitochondrial dysfunction in FABP3-overexpressing P19 cells in order to

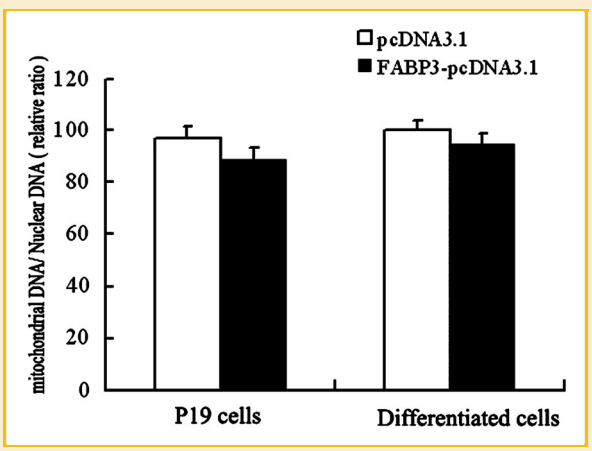

Fig. 5. Effect of fatty acid-binding protein 3 (FABP3) on the mitochondrial DNA copy number in differentiated P19 cells. Total DNA was extracted and relative expression levels of *cytB* copy number were determined by real-time quantitative polymerase chain reaction (qPCR) analysis with primers designed to target the *CYTB* RNA and were normalized to *28S rRNA* genes ($n = 6$). $P > 0.05$ compared with control cells (cells transfected with the empty vector).

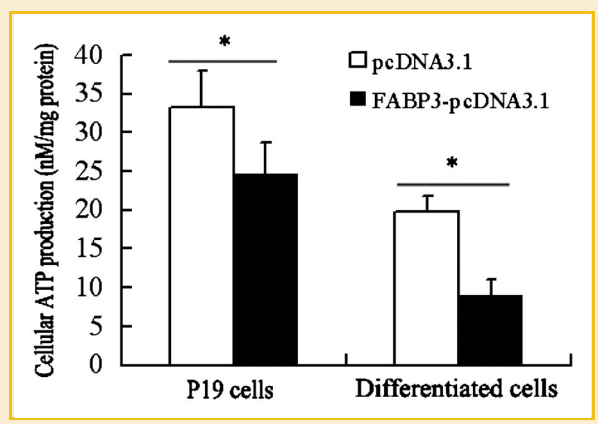

Fig. 6. Effect of fatty acid-binding protein 3 (FABP3) on cellular ATP production. Cellular ATP production was measured by a luciferase-based luminescence assay and was normalized to protein concentrations in differentiated P19 cells ($n = 6$). $^*P < 0.05$ compared with control cells. These data were based on four independent experiments. In the undifferentiatedP19 cells, the result was the same.

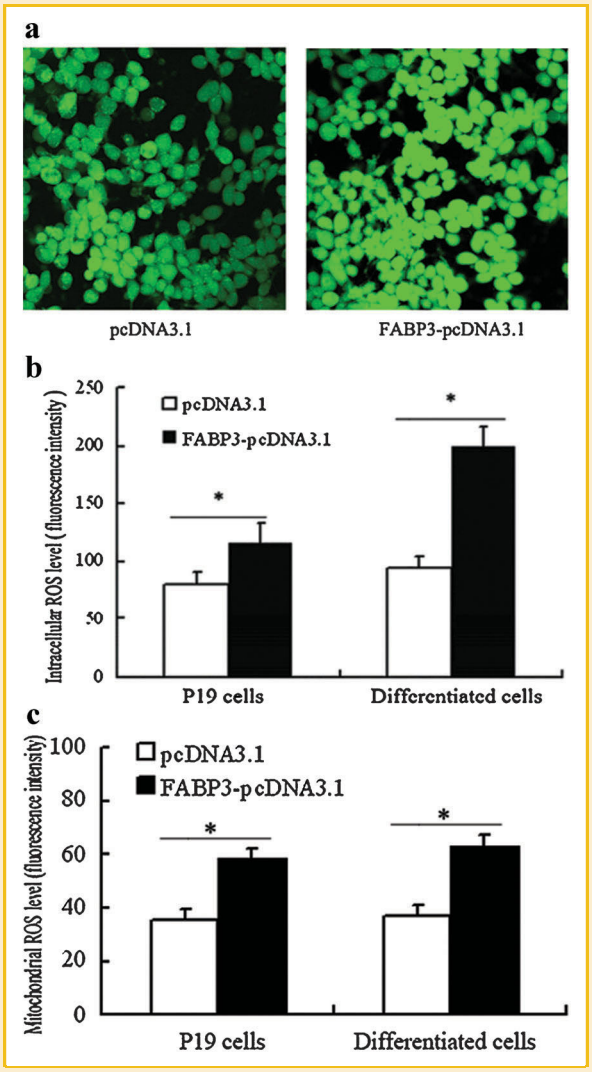

Fig. 7. Fatty acid-binding protein 3 (FABP3) increased intracellular levels of reactive oxygen species (ROS) in P19 cells. The ROS levels were determined by detection of the 2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (H_2-DCFDA) probe with a confocal laser scanning microscope (×200 magnification) (a); and a FACScan flow cytometer (excitation at 488 nm, emission at 530 nm) (b shows intracellular ROS, and c shows mitochondrial ROS). $^*P < 0.05$ compared with control groups.

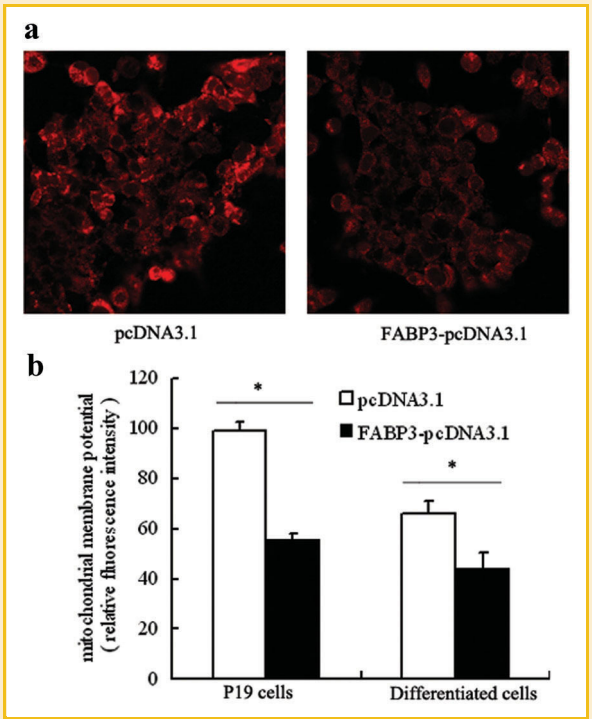

Fig. 8. Effect of fatty acid-binding protein 3 (FABP3) on mitochondrial membrane potential (MMP). The *FABP3*-overexpressing cells and cells transfected with empty vector control were stained with MitoTracker red, analyzed with a FACScan flow cytometer (excitation at 579 nm, emission at 599 nm), and then imaged using a confocal laser scanning microscope (×400 magnification) (a). $^*P < 0.05$ compared with control cells. Values for relative MMP are given (b). Values represent the mean ± standard deviation (SD) of three independent experiments.

determine whether this protein was involved in apoptosis and contributed to the mechanisms of apoptosis-related congenital cardiac malformations. The principal findings of the study are as follows: (1) FABP3 overexpression can inhibit proliferation and induce apoptosis in differentiated P19 cells; (2) FABP3 overexpression resulted in mitochondrion deformation. Mitochondria in FABP3-overexpressing P19 cells displayed compact and unclear cristae, but mitochondrial mtDNA were not changed; (3) ATP levels were reduced upon overexpression of FABP3; and (4) intracellular ROS levels increased and MMP was reduced in FABP3-overexpressing differentiated cells. These findings suggest that FABP3 overexpression has a detrimental effect on mitochondrion function in P19 cells.

Proliferation and apoptosis are presumed to play important roles during critical periods of heart development. Inadequate proliferation or excess apoptosis can directly or indirectly result in CHD [Fisher et al., 2000], which often results from altered proliferation and/or apoptosis of the septum, neighboring tissue, or myocardium [Oh and Schneider, 2002]. In the present study, we examined the potential influences of FABP3 on myocardial cell proliferation and apoptosis. Results suggest that FABP3 can promote myocardial cell apoptosis and inhibit proliferation. These results are consistent with earlier studies which suggest that Fabp3 inhibited proliferation and promoted apoptosis in undifferentiated P19 cells [Zhu et al., 2011]. This indicates that FABP3 may play an important role in the differentiation of cardiac myocytes.

Mitochondrial morphology is closely related to mitochondrion function and metabolic activity [Mannella, 2006]. Several reports have described an association between mitochondrion dysfunction and alterations in mitochondrion morphology [Yu et al., 2006; Katakam et al., 2007]. Despite the lack of a substantial decrease in the mtDNA copy number, our results demonstrated that overexpression of *FABP3* led to pronounced changes in mitochondrion morphology such as paracrystalline inclusions, typical of mitochondriopathies, vacuoles, swelling, twisted and condensed cristae, and hollow mitochondria with broken double membranes. The

change of mitochondrial function does not mean the change of mitochondrial number. Other unknown or undetected factors may play compensatory function in the mitochondrial number after mitochondrial impairment. More research needs to do to clarify this.

The mitochondrial matrix and cristae are the main sites for metabolism, and condensed mitochondria do not contain enough space to maintain normal or excessive metabolic needs. Therefore the mitochondrion morphological changes and dynamics perturbation prompted us to examine mitochondrion function. Most of the cell's supply of adenosine triphosphates (ATP) is generated by the mitochondria through the process of oxidative phosphorylation (OXPHOS). ATP production in mitochondria is coupled to the electron transport system in which the pumping of protons from the matrix into the intermembrane space generates an electrochemical gradient of protons that consist of a MMP and a pH gradient. The MMP is fundamental for the conversion of ADP to ATP via ATP synthase [Brown, 1992]. Our results showed that in *FABP3*-overexpressing P19 cells, a lower cellular ATP production was accompanied by a dramatic decrease in MMP. It is likely that the impaired mitochondria in the *FABP3*-overexpressing P19 cells will result in reduced intracellular ATP synthesis and MMP.

Mitochondria are also the major physiological sources of intracellular ROS because of the continuous generation of superoxide, a byproduct of the electron transport chain. High concentrations of ROS due to the imbalance between ROS production and removal can directly damage the mitochondrial proteins, DNA, and lipids in membrane components, and resulted in mitochondrion dysfunction [Choksi et al., 2004]. This damage primarily manifests as decreased mitochondrial ATP synthesis, dysregulation of intracellular lipid homeostasis, and induction of the mitochondrial permeability transition pores [Skulachev, 1999]. In our study, the levels of ROS in the *FABP3*-overexpressing P19 cells were significantly higher than that in the pcDNA3.1*myc*/His B vector control cells. The increased intracellular production of ROS coincides with the structural transition, as seen in the abnormal morphology of the mitochondrial cristae and membranes.

Finally, in relation to the correlation between apoptosis and mitochondrion impairment, it is likely that deformed mitochondria and elevated ROS induced by overexpression of *FABP3* may be contributors to cell apoptosis. As indicated above, mitochondrion dysfunction has emerged in *FABP3*-overexpressing P19 cells. Simultaneously, previous research has shown that apoptosis is mediated by two central pathways: the extrinsic (or death receptor) pathway and the intrinsic (or mitochondrion) pathway [Crow et al., 2004]. Here we focus on the mitochondria-dependent pathway. The mitochondria contain several highly lethal substances that can initiate apoptosis when released into the cytosol. One of the most important of these is the small electron transporter cytochrome *c*. Release of cytochrome *c* can be associated with opening of the permeability transition (PT) pore, a large nonselective channel in the outer mitochondrial membrane that is modulated by a redox-sensitive thiol and the redox state of pyridine nucleotide [Tanaka et al., 2002]. Our data demonstrated that *FABP3* overexpression increased significantly the production of ROS and decreased the MMP, therefore we may infer that increased ROS together with the decreased MMP result in opening of the PT pore; next cytochrome *c* is released from mitochondria and forms a complex with procaspase 9 and its cofactor apoptotic protease-activating factor-1 (APAF-1). Caspase 9 undergoes a conformational change that produces the active "apoptosome," which activates caspase 3 and executes the resulting apoptotic program [Bishopric et al., 2001]. Another proposed mechanism is that cytochrome *c* may be released directly from mitochondria due to swelling caused by rupture of the outer mitochondrial membrane [Li et al., 1997].

In summary, our results demonstrated that *FABP3* overexpression induced mitochondrion impairment in P19 cells during differentiation. This impairment was characterized by lower ATP synthesis and lower MMP, increased ROS level, and abnormal mitochondrion morphology. These findings may provide new insights into the mechanism of mitochondrion dysfunction in apoptosis. Furthermore, this gene may be a potential target in the treatment of apoptosis and apoptosis-related congenital cardiac malformations. Further studies in vivo are needed to elucidate the functions and mechanisms and to confirm whether abnormalities in *FABP3* expression contribute to mitochondrial dysfunction and apoptosis.

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REFERENCES

Andreyev AY, Kushnareva YE, Starkov AA. 2005. Mitochondrial metabolism of reactive oxygen species. Biochemistry (Mosc) 70:200–214.

Bajolle F, Zaffran S, Bonnet D. 2009. Genetics and embryological mechanisms of congenital heart diseases. Arch Cardiovasc Dis 102:59–63.

Besnard P, Niot I, Poirier H, Clement L, Bernard A. 2002. New insights into the fatty acid-binding protein (FABP) family in the small intestine. Mol Cell Biochem 239:139–147.

Bishopric NH, Andreka P, Slepak T, Webster KA. 2001. Molecular mechanisms of apoptosis in the cardiac myocyte. Curr Opin Pharmacol 1:141–150.

Brown GC. 1992. Control of respiration and ATP synthesis in mammalian mitochondria and cells. Biochem J 284(Pt 1):1–13.

Bruneau BG, Nemer G, Schmitt JP, Charron F, Robitaille L, Caron S, Conner DA, Gessler M, Nemer M, Seidman CE, Seidman JG. 2001. A murine model of Holt–Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. Cell 106:709–721.

Choksi KB, Boylston WH, Rabek JP, Widger WR, Papaconstantinou J. 2004. Oxidatively damaged proteins of heart mitochondrial electron transport complexes. Biochim Biophys Acta 1688:95–101.

Crow MT, Mani K, Nam YJ, Kitsis RN. 2004. The mitochondrial death pathway and cardiac myocyte apoptosis. Circ Res 95:957–970.

Fisher SA, Langille BL, Srivastava D. 2000. Apoptosis during cardiovascular development. Circ Res 87:856–864.

Hoye AT, Davoren JE, Wipf P, Fink MP, Kagan VE. 2008. Targeting mitochondria. Acc Chem Res 41:87–97.

Kaaman M, Sparks LM, van Harmelen V, Smith SR, Sjolin E, Dahlman I, Arner P. 2007. Strong association between mitochondrial DNA copy number and lipogenesis in human white adipose tissue. Diabetologia 50:2526–2533.

Katakam PV, Jordan JE, Snipes JA, Tulbert CD, Miller AW, Busija DW. 2007. Myocardial preconditioning against ischemia-reperfusion injury is abolished in Zucker obese rats with insulin resistance. Am J Physiol Regul Integr Comp Physiol 292:R920–R926.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479–489.

Lints TJ, Parsons LM, Hartley L, Lyons I, Harvey RP. 1993. Nkx-2.5: A novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development 119:419–431.

Mannella CA. 2006. Structure and dynamics of the mitochondrial inner membrane cristae. Biochim Biophys Acta 1763:542–548.

Maxwell DP, Wang Y, McIntosh L. 1999. The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. Proc Natl Acad Sci USA 96:8271–8276.

Nemer M. 2008. Genetic insights into normal and abnormal heart development. Cardiovasc Pathol 17:48–54.

Oh H, Schneider MD. 2002. The emerging role of telomerase in cardiac muscle cell growth and survival. J Mol Cell Cardiol 34:717–724.

Olson EN. 2006. Gene regulatory networks in the evolution and development of the heart. Science 313:1922–1927.

Poelmann RE, Gittenberger-de GA. 2005. Apoptosis as an instrument in cardiovascular development. Birth Defects Res C Embryo Today 75:305–313.

Qian Q, Kuo L, Yu YT, Rottman JN. 1999. A concise promoter region of the heart fatty acid-binding protein gene dictates tissue-appropriate expression. Circ Res 84:276–289.

Robin ED, Wong R. 1988. Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. J Cell Physiol 136: 507–513.

Singh MK, Li Y, Li S, Cobb RM, Zhou D, Lu MM, Epstein JA, Morrisey EE, Gruber PJ. 2010. Gata4 and Gata5 cooperatively regulate cardiac myocyte proliferation in mice. J Biol Chem 285:1765–1772.

Skulachev VP. 1999. Mitochondrial physiology and pathology; concepts of programmed death of organelles, cells and organisms. Mol Aspects Med 20:139–184.

Tanaka T, Hosoi F, Yamaguchi-Iwai Y, Nakamura H, Masutani H, Ueda S, Nishiyama A, Takeda S, Wada H, Spyrou G, Yodoi J. 2002. Thioredoxin-2 (TRX-2) is an essential gene regulating mitochondria-dependent apoptosis. EMBO J 21:1695–1703.

Tang MK, Kindler PM, Cai DQ, Chow PH, Li M, Lee KK. 2004. Heart-type fatty acid binding proteins are upregulated during terminal differentiation of mouse cardiomyocytes, as revealed by proteomic analysis. Cell Tissue Res 316:339–347.

Thompson CB. 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267:1456–1462.

van der Heyden MA, van Kempen MJ, Tsuji Y, Rook MB, Jongsma HJ, Opthof T. 2003. P19 embryonal carcinoma cells: A suitable model system for cardiac electrophysiological differentiation at the molecular and functional level. Cardiovasc Res 58:410–422.

Yu T, Robotham JL, Yoon Y. 2006. Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. Proc Natl Acad Sci USA 103:2653–2658.

Zhang H, Zhou L, Yang R, Sheng Y, Sun W, Kong X, Cao K. 2006. Identification of differentially expressed genes in human heart with ventricular septal defect using suppression subtractive hybridization. Biochem Biophys Res Commun 342:135–144.

Zhu C, Hu DL, Liu YQ, Zhang QJ, Chen FK, Kong XQ, Cao KJ, Zhang JS, Qian LM. 2011. Fabp3 inhibits proliferation and promotes apoptosis of embryonic myocardial cells. Cell Biochem Biophys 60:259–266.