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Silencing of FABP3 promotes apoptosis and induces mitochondrion impairment in embryonic carcinoma cells

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Abstract Fatty acid binding protein 3 (FABP3) (also known as H-FABP) is a member of the intracellular lipid-binding protein family, and is mainly expressed in cardiac muscle tissue. The in vivo function of FABP3 is proposed to be in fatty acid metabolism, trafficking, and cell signaling. Our previous study found that FABP3 is highly regulated in patients with ventricular septal defect (VSD), and may play a significant role in the development of human VSD. In the present study, we aimed to investigate the impact of FABP3 knockdown by RNA interference (RNAi) on apoptosis and mitochondrial function of embryonic carcinoma (P19) cells. The results revealed that downregulated FABP3 expression promoted apoptosis, and resulted in mitochondrial deformation, increased mitochondrial membrane potential (MMP), and decreased intracellular ATP synthesis. In addition, the knockdown of FABP3 also led to excess intracellular ROS production. However, there was no obvious influence on the amount of mitochondrial DNA. Collectively, our results indicated that FABP3 knockdown promoted apoptosis and caused mitochondrial dysfunction in P19 cells, which might be responsible for the development of human VSD.

Keywords Congenital heart disease · FABP3 · Mitochondria · RNA interference

Ya-Hui Shen and Gui-Xian Song are authors contributed equally to this work.

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Introduction

Congenital heart disease (CHD) present at birth is an important component of pediatric cardiovascular disease and constitutes a major percentage of clinically significant birth defects, with an estimated prevalence of 4 to 50 per 1,000 live births (Hoffman 1990; Moller et al. 1993). Cardiac development requires a precise and extremely complex series of molecular mechanisms to ensure the proper expression of cardiac transcription factors, and alterations in their expression can result in heart defects (Nemer 2008; Srivastava and Olson 2000). As a single cardiac malformation, ventricular septal defect (VSD) is the most common form of CHD and the most frequently encountered of congenital abnormalities (Sands et al. 1999). The ontogeny of ventricular septation is complex and requires cell-coordinated growth, specification, differentiation, migration and apoptosis, morphogenesis and cell–cell interaction. However, the precise molecular mechanisms that underlie VSD remain unknown.

In a previous study, we analyzed the gene expression profiles of VSD and identified 299 genes that were upregulated in VSD. Notably, fatty acid binding protein (FABP3) was upregulated simultaneously in VSD, which indicated that this protein may play a role in the cause of human VSD (Zhang et al. 2006). Further studies by our group revealed that overexpression of FABP3 inhibited proliferation, promoted apoptosis of embryonic myocardial cells, and affected the differentiation of cardiac precursors into mature cardiomyocytes (Zhu et al. 2011). The excessive apoptosis and inhibitive proliferation of the cells may lead to the disorder of the septa.

Apoptosis is the essential event of a vertebrate's organ formation and development process. Apoptosis not only participates in the development of the endocardia, epicardia, and neural crest, it is a fundamental aspect of morphology and also plays an important role in regulation. Too much or

no degree of apoptosis can cause congenital heart defects (Fiorina et al. 2004). Furthermore, it has been established that mitochondria play an important role in apoptosis under a variety of pro-apoptotic conditions (Green and Reed 1998).

In this study, we determined the effects of FABP3 knockdown on cell apoptosis and on the function of mitochondria in mouse embryonic carcinoma P19 cells in vitro. We chose to use P19 cells because they can undergo myocardial cell differentiation after treatment with 1 % dimethylsulfoxide (DMSO) (van der Heyden and Defize 2003). P19 cells may reflect the behavior of myocardial cells under these conditions.

Materials and methods

Cell culture and induction of differentiation

Mouse embryonic carcinoma (P19) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in α-MEM that contained 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin and cultured at 37 °C in an atmosphere of 5 % CO_2 in air. 293 T cells from the ATCC were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO™, Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10 % FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. P19 cell differentiation was induced using 1 % DMSO as described previously (Grepin et al. 1997). Briefly, P19 cells were grown in α-MEM supplemented with 10 % FBS until confluent. Two days after complete cell confluence occurred (designated as day 0), the cells were cultured in bacteriological dishes as aggregates in the presence of α-MEM that contained 10 % FBS and 1 % DMSO. At day 4, the cell aggregates were transferred to cell culture flasks. The medium was then switched back to α-MEM that contained 10 % FBS for an additional period of time as determined by the experimental design.

Construction of RNAi lentiviral vector and establishment of FABP3 silenced cells

The designed short hairpin RNA (shRNA) construct contained a unique 19 nt double-stranded FABP3 sequence that presented as an inverted complementary repeat, a loop sequence (5′-CTCGAG-3′), the RNA *Plo*III terminator (5′-TTTTTT-3′), and 5′ single-stranded overhangs for ligation into *Age*I- and *Eco*RI-digested pGLV–U6–Puro lentivirus vector (GenePharma, Shanghai, China). The recombinant vector was named pGLV–FABP3–shRNA. The negative control vector (pGLV–NC–shRNA) contained a nonsense shRNA insert in order to control any effects caused by non-RNAi mechanisms. We co-transfected the 293 T producer cell line with three optimized packaging plasmids (pGag/Pol, pRev and pVSV–G) and the pGLV–FABP3–shRNA or pGLV–NC–shRNA expression clone construct, which resulted in production of lentiviral stocks with a suitable titer. Stably transduced P19 cells were selected using puromycin, adding the minimum concentration of puromycin required to kill untransduced P19 cells. The efficiency of knockdown was detected by real-time quantitative polymerase chain reaction (qPCR). The sequences of the two cDNA fragments (sense strands) are as follows: FABP3, 5′-CATCCTGACTCTCACTCAT-3′; negative control, 5′-TTCTCCGAACGTGTCACGT-3′. The sequences of the primers are shown in Table 1.

Western blotting

Total proteins were isolated from cultured cells, separated on a 10 % gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes. These membranes were incubated with a monoclonal rabbit anti-cTnT antibody (CHEMICON, USA), and a monoclonal rabbit anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody (Proteintech Group, Inc., China), and goat anti-rabbit immunoglobulin G–horseradish peroxidase conjugate (Amersham, UK). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham).

Apoptosis assay

Cells were cultured in serum-free α-MEM for 24 h to induce apoptosis, and then harvested using trypsin at 0 and 24 h after replacement of the serum-free α-MEM with full medium that contained 10 % FBS, and washed with phosphate-buffered saline (PBS). The cells were centrifuged and resuspended in 1 mL binding buffer and then stained with 10 μL annexin V–FITC and 10 μL propidium iodide (PI) at room

Table 1 Oligonucleotide sequences for primer sets used in qPCR

Gene	Forward primer(5′–3′)	Reverse primer (5′–3′)
CYTB	TTTTATCTGCATCTGAGTTTAATCCTGT	CCACTTCATCTTACCATTTATTATCGC
28S	GGCGGCCAAGCGTTCATAG	AGGCGTTCAGTCATAATCCCACAG
FABP3	ATGAGAAGGAGGCGTGAC	ATCCCAACCCAAGAATGT
GAPDH	ATTCAACGGCACAGTCAA	CTCGCTCCTGGAAGATGG

for 15 min (Biovision, CA, USA). The stained cells were analyzed immediately using flow cytometry as described previously (Vermes et al. 1995a).

Electron microscopy

The transfected P19 cells were induced to differentiate as described above. On the 10th day of differentiation, the mature cardiomyocytes were collected after trypsin digestion, and fixed in a buffer that contained 2.5 % (v/v) glutaraldehyde and 4 % (v/v) paraformaldehyde. The cells were then washed in 0.1 M cacodylate buffer, postfixed with 1 % (w/v) osmium tetroxide and 1.5 % (w/v) potassium ferrocyanide for 1 h, washed in water, stained with 1 % (v/v) aqueous uranyl acetate for 30 min, and dehydrated through a graded series of ethanol to 100 %. The samples were then infiltrated and embedded in TAAB Epon (Marivac Canada Inc., St. Laurent, Canada). Ultrathin sections (60 nm) were cut on a Reichert Ultracut-S microtome, 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 RT-PCR for mitochondrial DNA

Relative amounts of mtDNA were determined by real-time qPCR as described previously (Kaaman et al. 2007). Briefly, DNA was isolated from differentiated cells with a DNA extraction kit (QIAGEN, Dusseldorf, Germany) and quantified by spectrophotometry at 260 nm. Two primer sets (listed in Table 1) were used for PCR analysis. A 110-nt mtDNA fragment within the *CYTB* gene was used for the 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 samples could be determined by real-time qPCR performed on an Applied Biosystems 7300 Sequence Detection System (ABI 7300 SDS; Foster City, CA, USA). A 291-bp region of the nuclear gene for 28 S was used to normalize results. The ratio of mtDNA to nuclear DNA reflected the concentration of mitochondria per cell.

Assessment of cellular ATP production

On the 10th day of differentiation, the ATP content of the P19 cells was measured using a luciferase-based luminescence assay kit (Biyuntian, Nantong, China). Briefly, differentiated P19 cells were homogenized in an ice-cold ATP-releasing buffer. ATP concentrations were then determined with a single-tube luminometer (Turner Biosystems, USA) and normalized to protein concentrations.

Confocal laser microscopy and flow cytometry

MitoTracker, a red mitochondrion-specific cationic fluorescent dye (Molecular Probes, Invitrogen, Carlsbad, CA, USA), was used to evaluate the mitochondria membrane potential (MMP) (Maxwell et al. 1999). A 2′, 7′-dichlorodihydrofluorescein diacetate acetyl ester (H2-DCFDA) probe (Sigma, St. Louis, MO, USA) was used to estimate the intracellular ROS levels (Maxwell et al. 1999). The cells were incubated with 150 nM of MitoTracker and 5 μM of H2-DCFDA for 30 min at 37 °C, washed three times with pre-warmed PBS and then imaged using a confocal laser scanning microscope (excitation at 579 nm, emission at 644 nm, ×400 magnification). For flow cytometry, the cells were trypsinized and centrifuged at 500 rpm at 4 °C for 5 min, and then resuspended in PBS buffer (pH 7.4). The cells were analyzed with a FACScan flow cytometer using the CellQuest software (BD Biosciences, San Jose, CA, USA).

Statistical analysis

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, USA). All values were presented as means±standard error of the mean (SEM) from at least three independent experiments. The threshold of statistical significance was defined as $P<0.05$.

Results

Appraisal of FABP3 suppression efficiency and cell differentiation

The knockdown efficiency of FABP3 was evaluated by real-time PCR. The relative FABP3 mRNA level in cells was normalized against the mRNA levels of an endogenous control gene, GAPDH. The value of FABP3 mRNA in the cells infected with pGLV–NC–shRNA was set as the negative control group. As shown in Fig. 1a, cells infected with pGLV–FABP3–shRNA showed significantly reduced transcription of FABP3 mRNA when compared with the negative control (*$P<0.05$). The FABP3 mRNA level of the pGLV–FABP3–shRNA-infected cells was 24 % that of the negative control cells. To investigate the differentiation of P19 cells into mature cardiomyocytes, we used western blotting to identify the expression of cTnT protein during differentiation. In Fig. 1b, we show the increase in cTnT expression.

Effects of FABP3 silencing on cell apoptosis

Annexin V–FITC binds to phosphatidylserine and can be used to detect the early stages of apoptosis (Vermes et al. 1995b).

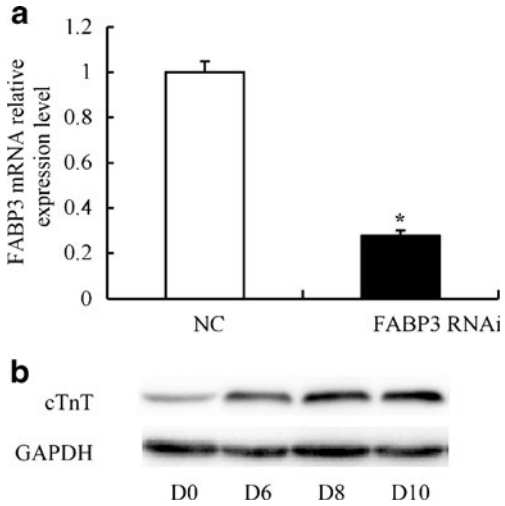

Fig. 1 Appraisal of FABP3 suppression efficiency and cell differentiation. **a** Fatty acid binding protein 3 (FABP3) mRNA relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the pGLV–FABP3–shRNA-transfected differentiated P19 cells was examined by real-time PCR in comparison with pGLV–NC–shRNA-transfected cells. The FABP3 mRNA inhibitory efficiency was 76 % (*P<0.05 vs. negative control (NC) cells). The results represent the mean±standard deviation (SD) of six experiments. **b** To investigate the differentiation of P19 cells into mature cardiomyocytes, we used western blotting to identify the expression of cTnT protein during differentiation

Apoptotic cells (0 and 24 h after replacement of FBS-free α-MEM with full medium) were quantified by flow cytometric analysis after staining with annexin V–FITC. This result showed that FABP3 knockdown promoted cell apoptosis when the cells were induced by serum deprivation (** P<0.05) (Fig. 2).

Effects of FABP3 knockdown on mitochondrion morphology and mtDNA copy number in P19 cells

We monitored the ultrastructure of mitochondria and assessed the mtDNA copy number in FABP3 knockdown cells, which represented the relative quantity of mitochondria present. As shown in Fig. 3a, the mitochondria in FABP3 knockdown cells were smaller in size and had an abnormal condensed morphology. Signs of mitochondrion damage ranged from swelling and reduced density to almost completely hollow mitochondria with broken double membranes. The mtDNA copy number per mitochondrion is considered generally to be constant in all mammalian cell types (Robin and Wong 1988). Using real-time PCR, we determined the mtDNA copy number, and showed that there was no significant difference in mtDNA copy number between the two groups (P>0.05) (Fig. 3b).

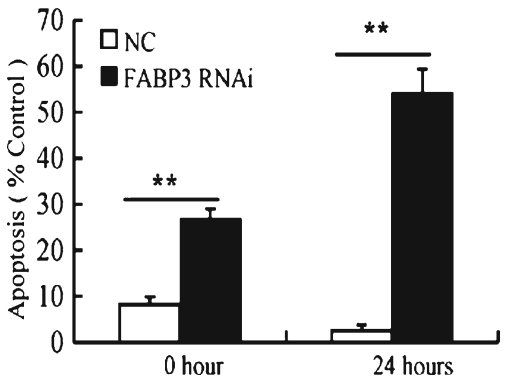

Fig. 2 Effect of FABP3 knockdown on cell apoptosis. Apoptosis was assayed by binding by annexin V–FITC. Suppression of fatty acid binding protein 3 (FABP3) can promote apoptosis induced by serum deprivation in P19 cells. The results represent mean±standard deviation (SD) of six independent experiments (**P<0.05)

Cellular ATP production was decreased upon FABP3 knockdown

In eukaryotic cells, the mitochondrion is the major platform for energy transduction, and produces ATP via oxidative metabolism of nutrients. Impaired mitochondria may lead to a lack of ATP. We found that the total cellular ATP production was decreased dramatically in FABP3 silenced cells (Fig. 4).

Effects of FABP3 knockdown on intracellular ROS levels and MMP

We next sought to determine whether FABP3 knockdown affects ROS content and MMP. ROS levels in FABP3

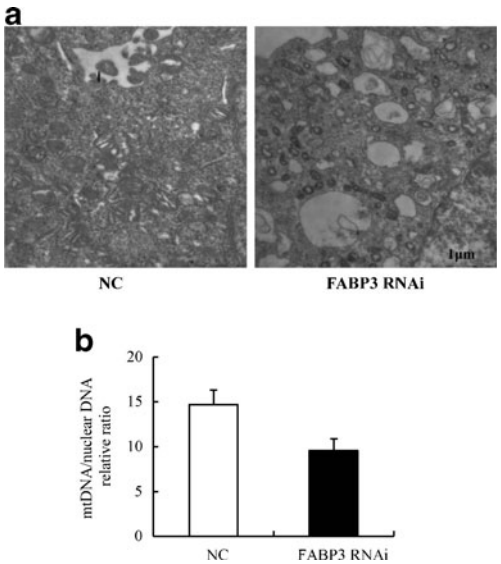

Fig. 3 Effects of fatty acid binding protein 3 (FABP3) knockdown on mitochondrion morphology and the mitochondrial DNA (mtDNA) copy number. **a** Ultrastructural visualization of mitochondria in P19 cells was performed using transmission electron microscopy (less than×30,000 magnification). The results were representative of three independent experiments. The scale bars in the bottom right hand corner of (a) represents 1 μm. **b** On the 10th day of differentiation, cellular mtDNA content was assessed by real-time quantitative polymerase chain reaction (PCR) analysis with primers designed to target the *CYTB* and *28 S rRNA* genes (n=6). P>0.05 in comparison with negative control (NC) cells

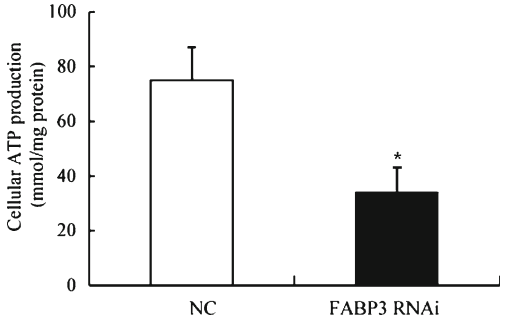

Fig. 4 Effects of fatty acid binding protein 3 (FABP3) knockdown on intracellular ATP levels. Total cellular ATP levels were measured using a luciferase-based luminescence assay kit. The results are representative of those values obtained from three independent experiments (n=6). Values represent the mean±standard deviation (SD). *P<0.05

knockdown cells were much higher than that of control cells, as indicated by more intense fluorescence signals in the presence of the compound dichlorodihydrofluorescein diacetate acetyl ester (DFCDA)(Fig. 5a). We found that FABP3 silencing resulted in an elevation in MMP (Fig. 5b), as shown by the potential dependent fluorescence probe. These results clearly demonstrated that FABP3 silencing induced apoptosis that was accompanied by mitochondrial dysfunction in P19 cells.

Discussion

CHD is a multifactorial disease that results from the interactions between susceptibility genes and environmental factors. Nevertheless, the genetic basis of the majority of CHD remains unknown. To date, only a few genes or markers of critical regulators of heart formation have been identified as being associated with CHD; these genes include *NKX2.5* (Lints et al. 1993), *Tbx5* (Bruneau et al. 2001), and *GATA4* (Watt et al. 2004). However, the basic pathogenetic mechanism of VSD remains unclear.

Previous studies have shown that FABP3 is upregulated during terminal differentiation of mouse cardiomyocytes (Tang et al. 2004). McCann et al. found that FABP3 could be used as a novel biomarker in the early diagnosis of acute ischemic chest pain (McCann et al. 2008). We found that FABP3 is highly regulated in VSD patients, compared with normal controls, using subtractive hybridization experiments (Zhang et al. 2006). These findings suggested that FABP3 may play a role of VSD formation. Thus, we sought to determine whether FABP3 was associated with cardiac abnormalities in humans.

It is well known that P19 cells are pluripotent when exposed to DMSO and that they can differentiate into embryonic myocardial cells. Therefore this cell line is a useful

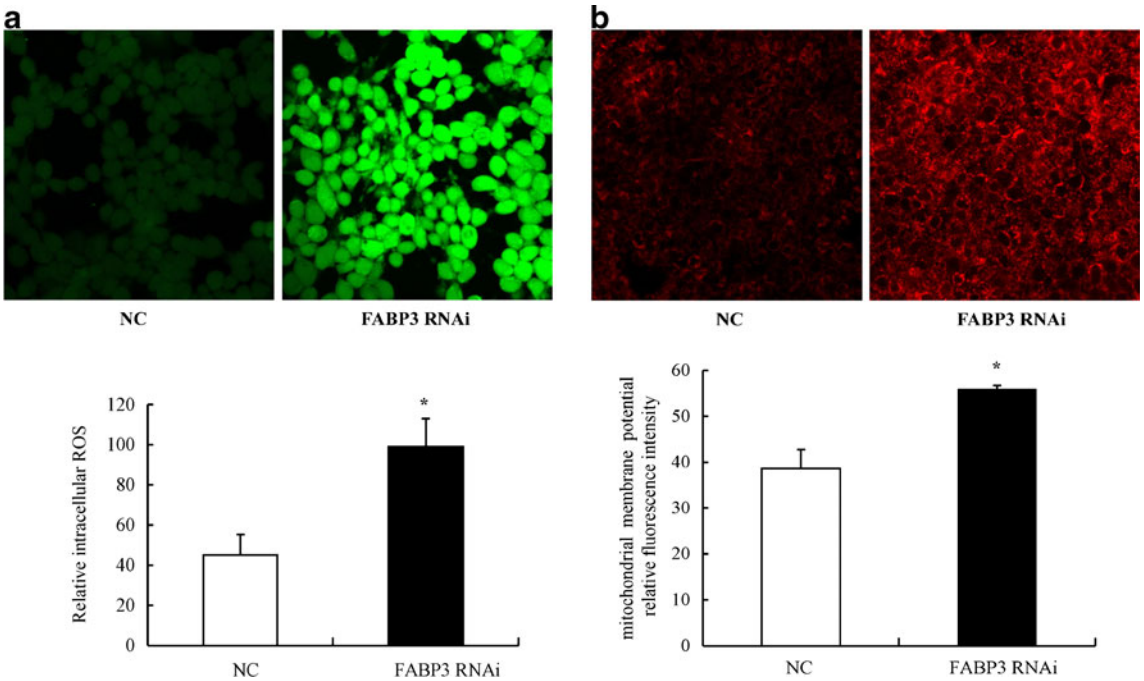

Fig. 5 Effects of fatty acid binding protein 3 (FABP3) silencing on intracellular reactive oxygen species (ROS) content and mitochondrial membrane potential (MMP) in differentiated cells. The ROS levels and MMP were determined on the 10th day of differentiation, using FACScan flow cytometer (excitation at 488 nm, emission at 530 nm), and then imaged using a confocal laser scanning microscope (n=6). **a** ROS levels were determined by measuring the fluorescence of DCFDA using a confocal laser scanning microscope and by fluorescence activated cell sorting (FACS) analysis. *P<0.05 vs. negative control (NC) cells. **b** MMP was determined by measuring the fluorescence of Mito-Tracker red using a confocal laser scanning microscope and by FACS. *P<0.05 vs. the NC cells

model system that reflects the in vivo biological features of myocardial cells (van der Heyden and Defize 2003; van der Heyden et al. 2003). Accordingly, we chose this system by which to investigate the effects of FABP3 silencing on cell apoptosis and mitochondrion function.

RNAi is a powerful gene-silencing mechanism that operates in most eukaryotic cells (Aagaard and Rossi 2007). The effector molecules are comprised of short duplex RNA sequences of 19–23 nt that direct inhibition of homologous genes. This natural pathway is important for processing of regulatory microRNAs. In this study, we successfully constructed an RNAi lentiviral vector to suppress endogenous FABP3 expression. We found by real-time PCR that co-transduction resulted in a significant reduction in *FABP3* gene transcription. These effects were not observed in the cells that were infected with the negative control shRNA when compared with normal cells.

Our results demonstrated that the inhibition of FABP3 expression promoted cell apoptosis. The molecular mechanism that underlies this effect still remains unknown. Michael et al. suggested that mitochondria play an important role in transmission and amplification of apoptosis signals (Crow et al. 2004). Therefore, in this study, we further investigated the effect of FABP3 knockdown on mitochondrion function in P19 cells. We found that suppression of FABP3 expression caused mitochondria dysfunction. The major findings were as follows: (i) mitochondria in FABP3-silenced cells had compact and unclear cristae; (ii) intracellular ROS and MMP increased, and ATP contents were reduced; and (iii) the knockdown of FABP3 had no obvious effect on the level of mitochondrial DNA.

Mitochondria are a major source of ROS, with up to 2 % of oxygen consumed by mitochondria converted to superoxide. There is considerable evidence to suggest that oxidative damage plays a role in mitochondria dysfunction (Beal 2003). Mitochondria morphology is closely related to mitochondria function and metabolic activity (Mannella 2006). The presence of condensed mitochondria indicated that ATP production had decreased and this phenomenon was shown in our study. It has been reported that mitochondria morphology is controlled by MMP across the inner membrane (Legros et al. 2002). These observations appear to contradict other findings. We speculate that the reasons for the observed phenomenon are as follows: FABP3 knockdown cells generate more ATP in order to compensate for lost ATP production, and an elevation of MMP is known to elevate the production of ROS. In summary, the present findings revealed that FABP3 may be a useful target for regulation of myocardial cell development. Defects in mitochondria mediated apoptosis may stimulate the development of VSD.

Although important advances have been made in diagnostic evaluation and therapeutic strategies for heart defects, various cardiac malformations still lead to significant morbidity and mortality in humans. Therefore, the next step in this study would be to determine whether FABP3 is associated with cardiac abnormalities in humans.

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