Am J Transl Res 2016;8(2):644-652
www.ajtr.org /ISSN:1943-8141/AJTR0014072

Original Article

Prolactin mediates effects of chronic psychological stress on induction of fibrofatty cells in the heart

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Received August 6, 2015; Accepted December 29, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: Cardiocyte apoptosis plays an important role in the pathogenesis of heart diseases. The mechanism is unclear. It is reported that prolactin (PRL) is involved in cardiac disorders. This study aims to investigate the role of PRL in mediating the psychological stress-induced fibrofatty cell differentiation in the heart. In this study, BALB/c mice were treated with a 30-day restraint stress. The heart tissue was processed by paraffin embedding and hematoxylin and eosin. The expression of Sca1 in NIH3T3 cells was assessed by cell culture, flow cytometry and Western blotting. The results showed that chronic stress induced fibrofatty cells in the mouse heart and high serum PRL levels. The induction of fibrofatty cell was mimicked by administration with recombinant PRL. The stress also induced the expression of Sca1 in the mouse heart. Exposure of NIH3T3 cells (a fibroblast cell line) to PRL in the culture enhanced the expression of stem cell antigen-1 (Sca1), phosphorylation of signal transducer and activator of transcription 3 (STAT3) and expression of adipocyte-related protein molecules, including adiponectin, fatty acid binding protein (aP2), peroxisome proliferator activated receptor-g (PPARg) and CCAAT/enhancer binding protein (C/EBP)α, in the cells. We conclude that psychological stress-derived PRL induces fibroblasts to differentiate into fibrofatty cells in the heart.

Keywords: Heart, chronic stress, apoptosis, prolactin, fibrofatty cells

Introduction

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a cardiac muscle disease; it is also suggested to term as the arrhythmogenic cardiomyopathy because both ventricles can be affected [1]. ARVC is one of the cardiac diseases causing sudden death [2]. The prevalence of ARVC is about 1/3000 to 1/5000 in the general population [3]. It is reported that the homozygous loss of function mutations in desmoplakin in patients with ARVC [4]. Desmoplakin is the first desmosomal gene linked to autosomal dominant ARVC following the identification of a missense mutation in desmoplakin in a family of Italian descent harboring ARVC [5]. However, current mouse models targeting loss or mutations in desmoplakin [6, 7] do not recapitulate the postnatal severity and pathogenic hallmarks of the disease as seen in patients [4]. To date, the pathogenesis of ARVC remains largely unknown.

ARVC is characterized by a progressive right ventricle fibrofatty replacement and the presence of inflammatory infiltrates in myocardium; T-lymphocytes are the main infiltrate cell types [8], which implies that immune dysregulation may be involved in the pathogenesis of ARVC. However, further evidence is lacking.

Psychological stress is one of the factors inducing immune dysfunction [9] as well as inducing cardiac lesions [10]. Meta-analysis indicates that mental stress is involved in the pathogenesis of myocardial ischemia and subsequent cardiac events in patients with coronary artery disease [11]. Animal studies have revealed a molecular basis for the higher risk of cardiovascular disorders in patients with posttraumatic stress disorders, which suggests the likelihood of cardiac dysfunction induced by long-term stress exposures [12]. One of the major mediators of psychological stress is the prolactin (PRL) [13]. Fibroblasts express the PRL receptor

(PRLR) [14]. Besides its role in lactation and reproduction, PRL is also involved in various bio-activities, such as in the energy metabolism by modulating the activities of the pancreas and the adipose tissue [15]. Considering the increase in the fibrofatty cells in the ventricular tissue of ARVC, we hypothesize that chronic psychological stress-released PRL induces fibrofatty cells in the heart. In this study, we treated mice with a 30-day chronic restrain stress. The results showed that abundant fibrofatty cells were induced in the cardiac ventricular tissue, which was abolished by the antagonist of PRL. Treating the NIH3T3 cells (a fibroblast cell line) with PRL generated fibrofatty cells.

Materials and methods

Reagents

2-Hydroxy-4-((((4-methylphenyl) sulfonyloxy) acetyl) amino)-benzoic acid (S3I-201), antibodies of PRL (C-20), PRL-R1 (Q-20), PRL-R2 (N-20), Sca1 (C-20), STAT3 (C-20), collagen type I and STAT3-p (Ser-727) were purchased from Santa Cruz (Beijing, China). The FITC-anti-Sca1 antibody was purchased from BD Biosciences (Beijing, China). The cabergolin was purchased from Sigma Aldrich (Beijing, China). The mouse PRL was synthesized by ChinaPeptide Co., Ltd. (Shanghai, China). The ELISA kits of CRH and PRL were purchased from Biomart (Shanghai, China).

Mice

Male BALB/C mice (8-10 week old) were purchased from The Experimental Center of Beijing Fuwai Hospital. The mice were maintained in a pathogen-free environment with free access to food and water, and were housed under a 12 h light/dark cycle in a temperature-controlled room (22 ± 2°C). The experimental procedures were approved by the Animal Ethic Committee at Fu Wai hospital. The experiments were performed in accordance with the guidelines.

Heart samples from human ARVC subjects

Heart samples were collected from 10 ARVC patients (5 males; 5 females; the patients were undergone heart transplantation). The samples were processed for collagen immune staining. The using human tissue in the present study was approved by the Human Ethic Committee at Fu Wai hospital. An informed, written consent was obtained from each patient. The experiments involving human subjects, were carried out in accordance with the approved guidelines.

Chronic restraint stress

Following published procedures, mice were treated with restraint stress daily for 30 days. A mouse was placed in a 50 ml plastic tube (30 mm diameter × 115 mm length) with 8 holes (5 mm in diameter of each hole) for 2 h (8 am-9 am and 8 pm-9 pm respectively) at room temperature. The sham stress control mice were left in their cage.

Western blotting

The total proteins were extracted from heart tissue or cells. The proteins were fractioned by SDS-PAGE and transferred onto a PVDF membrane. After blocking with 5% skim milk for 1 h, the membrane was incubated with the primary antibodies (300 ng/ml) overnight at 4°C, and followed by the secondary antibodies (labeled with horseradish peroxidase). Washing with TBST (Tris-buffered saline Tween-20) was performed after each incubation. The immune complex on the membrane was developed with ECL. The results were photographed with a KODAK Image Station 4000 Pro (KODAK, Shanghai, China). The integrated density of the Western blots was determined by software ImageJ and normalized to a percentage of the internal control β-actin.

Immunohistochemistry

The ventricular tissue was frozen with liquid nitrogen; the cryosections were fixed with cold acetone, blocked with 1% bovine serum albumin and incubated with antibodies of collagen (1:300) or an isotype IgG (control) at 4°C overnight. After washing with phosphate buffered saline (PBS), the sections were incubated with the secondary antibody (labeled with HRP) for 1 h at room temperature. The sections were washed with PBS, stained with hematoxylin, mounted with cover slips, and observed with a light microscope. The section slides were coded; the observers did not aware the code to avoid the observer bias.

Table 1. Primers using in RT-qPCR assay

Molecules	Forward	Reverse
Sca1	ttcttgtggccctactgtgt	gggcagatgggtaagcaaag
Collagen	cctgacaagttccaggggat	ccttgtgtgacttgctgctt
Adiponectin	ggtcctaagggtgagacagg	agtcccggaatgttgcagta
aP2	atgtcagcaggaagtcacca	aaccctgccaaagagacaga
PPARγ	agggcgatcttgacaggaaa	cgaaactggcacccttgaaa
C/EBPα	gcaagccaggactaggagat	aatactagtactgccgggcc
β-actin	gtgggaatgggtcagaagga	tcatcttttcacggttggcc

Real time quantitative RT-PCR (RT-qPCR)

The total RNA was extracted from the cells with the TRIzol reagent. The cDNA was synthesized with a reverse transcription kit. qPCR was performed with a Mini Opticon RT-qPCR device (Bio-Rad, Shanghai, China) with the SYBR Green Master Mix. The results were calculated with the $2^{-\Delta\Delta Ct}$ method and normalized to a percentage of the internal control of β-actin. The primers using in the present study are presented in **Table 1**.

Cell culture

NIH3T3 cells (a fibroblast cell line; purchased from ATCC, USA) were cultured in Claycomb medium (Sigma-Aldrich, Beijing, China) according to the manufacturer's suggestions. PRL was added to the culture medium at a concentration of 100 ng/ml [16]; control cells were cultured with medium alone. The medium was changed in 3-5 days.

Flow cytometry

Cells were fixed with 2% paraformaldehyde containing 0.1% Triton X-100 for 2 h at room temperature. After washing with PBS, the cells were blocked with 1% BSA for 30 min and incubated with FITC-labeled antibodies for 1 h. After washing with PBS, the cells were analyzed with a flow cytometer (FACSCanto II, BD Bioscience). The data were analyzed by software FlowJo.

Enzyme-linked immunosorbent assay (ELISA)

The blood was collected from each mouse at sacrifice; the sera were isolated. The levels of PRL in the sera were determined by ELISA with a commercial reagent kit following the manufacturer's instructions.

Echocardiography

Echocardiographic measurements were performed on the mice under light anesthesia with tribromoethanol-amylene hydrate (2.5% wt/vol, 8 μl/g i.p.), as described previously [17]. Briefly, two-dimensional parasternal short-axis views were obtained at the levels of the papillary muscles. The transducer was properly placed. Two-dimensional targeted M-mode tracings were recorded.

Human ARVC heart samples

This study consisted of 32 patients undergone heart transplantation (22 men and 10 women; ages 19 to 75 years); specimens from the right ventricles were examined. The diagnosis of ARVC was made by their physicians, according to the diagnostic criteria of ARVC [18]. The using human tissue in this study was approved by the Human Ethic Committee at Fuwai Cardiovascular Disease Hospital. An informed consent was obtained from each patient.

Statistics

The data are presented as mean ± SD. The difference between groups was determined by ANOVA. A $p<0.05$ was set as a significant criterion.

Results

Chronic stress induces fibrofatty cells in the mouse heart

It is suggested that psychological stress is associated with heart diseases [19]; the underlying mechanism is to be further investigated. In the first step of the present study, we treated BALB/c mice with a 30-day chronic restraint stress. The heart tissue was observed by immunohistochemistry. A large number of adipocyte-like cells were observed in mice treated with stress, those cells were collagen-positive, indicating they are fibrofatty cells. Although few fibrofatty cells could be seen in the naive mouse heart tissue (**Figure 1A**), the stressed mice showed the fibrofatty cells in the heart (**Figure 1B**), which did not occur in the mice treated with sham stress (**Figure 1C**). The stressed mice also showed an abnormal electrocardiogram with an unexplained arrhythmia

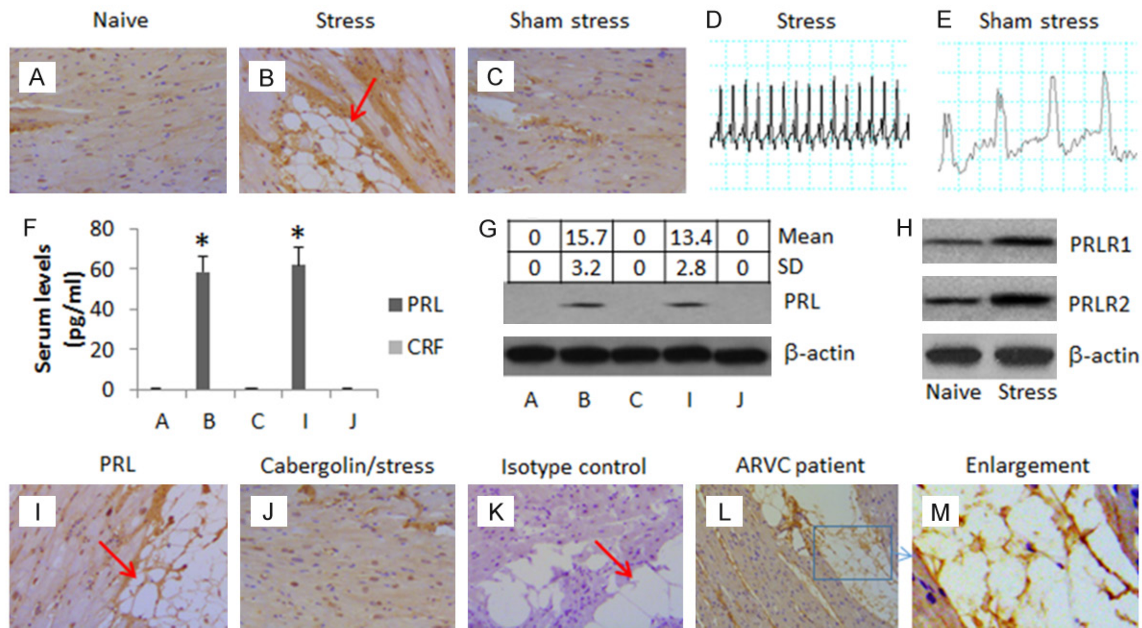

Figure 1. Stress induces ARVC-like lesions in the mouse heart. BALB/c mice (6 mice per group) were treated with a 30-day restraint stress, or sham stress, or PRL (10 ng/mouse in 0.1 ml saline, i.p. twice a day, 8 am and 8 pm), or stress, plus cabergolin (an antagonist of PRL; 500 μg/Kg body weight in 0.1 ml saline). (A-C and I-K) the representative images (× 100) show the positive staining (in brown) of collagen in mouse heart sections. The cell membrane of the fibrofatty cells was collagen positive. (K) is a negative control (stained with isotype IgG). The red arrows point to the fibrofatty cells. (D and E) the electrocardiograms of mice treated with stress (D) and sham stress (E). (F) the bars indicate the PRL levels in the sera of the mice (determined by ELISA) (mean ± SD; *, $p<0.01$, compared with group A). (G) the Western blots indicate the PRL levels in the mouse heart tissue. (H) the Western blots indicate the PRLR levels in the heart tissue. The tables above the Western blots (G) show the integrated density of the blots. The group labels of (F and G) are the same as the image labels. The data represent 6 independent experiments. (L) a representative image of the ARVC heart from 10 patients. (M) an enlargement of the selection in (L).

and T-wave inversion (**Figure 1D**), which did not occur in the sham-stressed mice (**Figure 1E**).

Published data suggest that PRL [20, 21] and corticotropin releasing factor (CRF) [22] are mediators of psychological stress; thus, we measured the levels of PRL and CRF in the mouse serum and in the heart tissue. The results showed that the levels of both PRL and CRF were below the detectable levels in the sera and heart tissue of normal control mice. After treatment with stress, marked increases in the levels of PRL, but not CRF, were detected in the sera (**Figure 1F**) and in the heart tissue (**Figure 1G**). The PRL receptor (PRLR) was also detected in the heart tissue of naïve mice, which was markedly increased after the 30-day stress (**Figure 1H**). The results suggest that psychological stress induces PRL release in the peripheral system, including the serum and the heart.

The above results implicate that psychological stress increases the production of PRL; it is possible that the fibrofatty cells in the heart are induced by PRL. To test the inference, we treated mice with PRL daily for 30 days. It also induced the fibrofatty cells in the heart (**Figure 1I**). To strengthen the results, we performed an experiment with restraint stress in the presence of a PRL antagonist. Indeed, the stress-induced the fibrofatty cells in the heart were abolished (**Figure 1J**). In addition, we collected heart samples from 10 patients with ARVC. A large number of fibrofatty cells also identified in the heart tissue sections from 8 (80.0%) patients (**Figure 1L**, **1M**). In the collection, the results suggest that the stress-derived PRL plays an important role in the induction of the fibrofatty cells in the heart.

Psychological stress increases the expression of stem cell antigen-1 (Sca1) in the heart

Sca1 is a critical molecule to drive stem cells to differentiate into adipocytes [23]. We wondered if the expression of Sca1 was up regulated in the heart of mice with fibrofatty cells after treat-

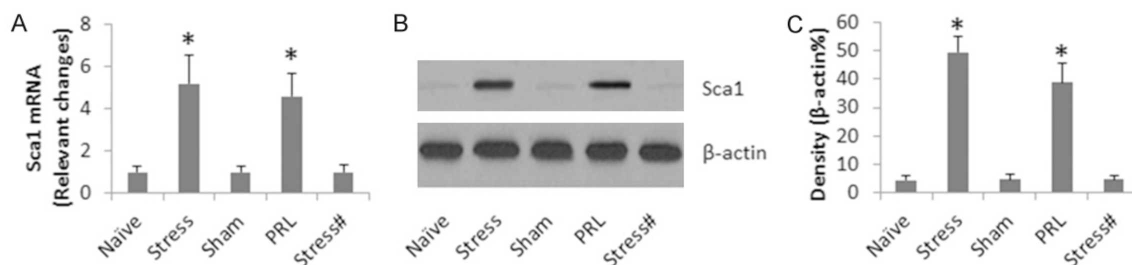

Figure 2. Stress increases Sca1 levels in the mouse heart. Mice (6 mice per group) were treated with the 10-day restraint stress or sham stress as denoted on the X axis of (A). The bars in (A) show the mRNA levels of Sca1. The Western blots in (B) show the protein levels of Sca1. (C) The bars indicate the integrated density of the immune blots of (B). #, PRL inhibitor (cabergolin; 500 mg/kg body weight; ip, 30 min before each stress session). The data are presented as mean ± SD; *, p<0.01, compared with naïve group. The data represent 6 independent experiments.

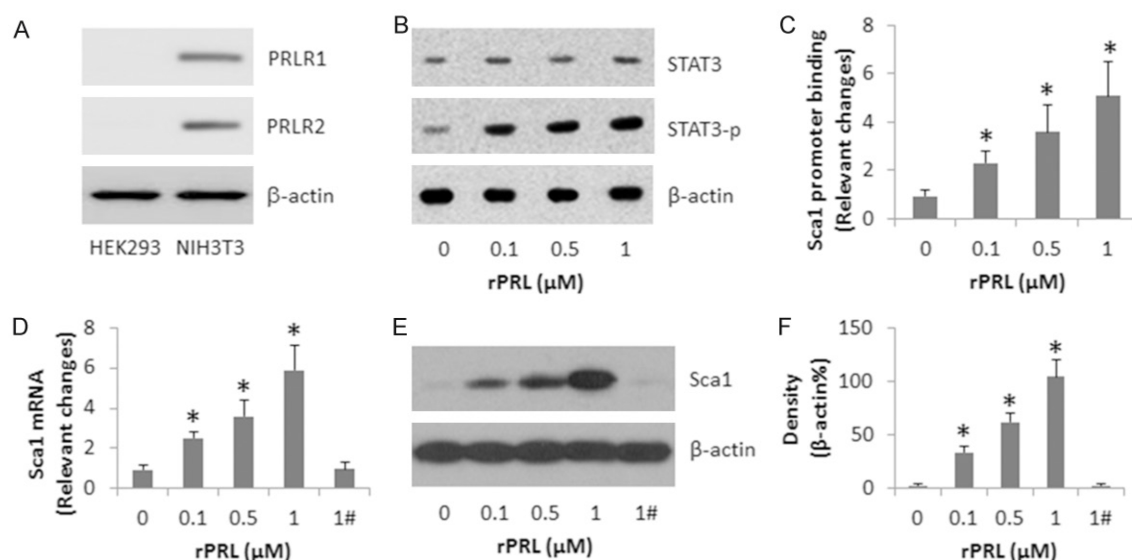

Figure 3. PRL induces Sca1 expression in NIH3T3 cells. NIH3T3 cells were cultured in the presence of PRL at gradient concentrations for 5 days. The cell extracts were analyzed. A. The Western blots indicate the protein levels of PRL receptor (PRLR) in HEK293 cells (using as a negative control) and NIH3T3 cells. B. The IP results indicate the protein levels of STAT3 and pSTAT3 in NIH3T3 cells. C. The ChIP results indicate the Sca1 promoter binding by pSTAT3. D. The bars indicate the mRNA levels of Sca1. E. The Western blots indicate the protein levels of Sca1. F. The bars indicate the summarized density data of the blots of E. #: The presence of S31-201 [An inhibitor of STAT3 (100 pg/ml)]. The data are presented as mean ± SD. *, p<0.01, compared with group "0". The data represent 3 independent experiments.

ing with stress. To this end, we assessed the levels of Sca1 in the heart tissue obtained from the mice treated with the 30-day restraint stress. The results of Western blotting showed that Sca1 was barely detected in the wild mouse heart tissue, but significantly increased after the 30-day stress, which did not occur in mice treated with sham stress. To treat mice with PRL also increased the levels of Sca1 in the heart tissue. In addition, the presence of a PRL inhibitor abolished the stress-induced Sca1 in the heart tissue (**Figure 2**). The results suggest that PRL is the factor mediating the effect of stress on inducing Sca1 expression in the heart tissue.

PRL modulates the expression of Sca1 in fibroblasts

To strengthen the data of **Figure 2**, we cultured NIH3T3 cells (a cell line of mouse fibroblast) in the presence of PRL in the culture for 5 days. The results showed that the NIH3T3 cells expressed PRL receptors (**Figure 3A**). It is

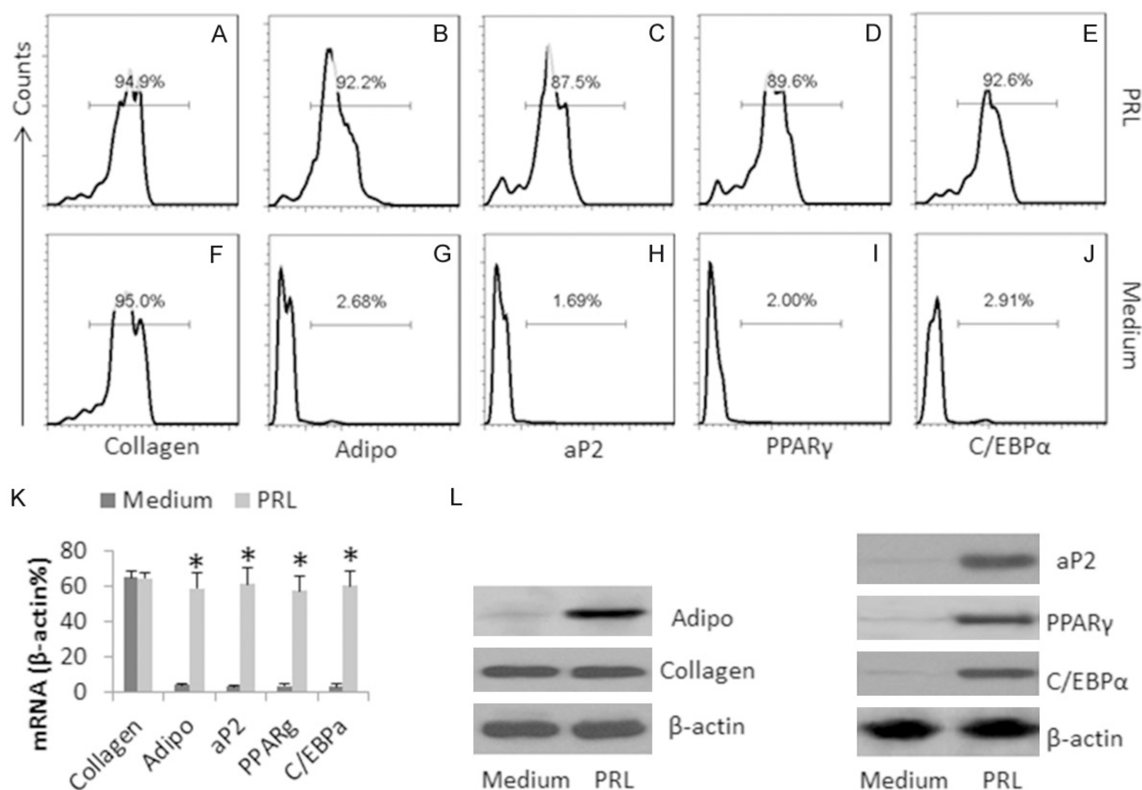

Figure 4. PRL induces fibrofatty cell differentiation. NIH3T3 cells (a fibroblast cell line) were cultured in the presence of PRL (100 ng/ml), or cultured with medium alone, for 4 weeks. The cells were analyzed by flow cytometry (A-J), RT-qPCR (K), and Western blotting (L). (A-J) the histograms show the positive stained cells as denoted below each subpanel. (K) the bars indicate the mRNA levels (mean ± SD; *, $p<0.01$, compared with the medium group). (L) the Western blots show the protein levels in the cells. Adipo = adiponectin. The data are representatives of 3 independent experiments.

reported that STAT3 is involved in the downstream activities of PRL [24]. We wondered if STAT3 was also involved in the PRL-induced Sca1 expression in NIH3T3 cells. To this end, we analyzed the STAT3 phosphorylation in the NIH3T3 cells. The results showed that the exposure to PRL markedly increased the levels of phosphorylated STAT3 in the NIH3T3 cells (**Figure 3B**). Further results showed that the pSTAT3 bound to the Sca1 promoter in a PRL dose-dependent manner (**Figure 3C**), which resulted in enhancement of the Sca1 expression in the NIH3T3 cells (**Figure 3D-F**). To enforce the results, we treated the NIH3T3 cells with PRL and a STAT3 inhibitor in the culture. Indeed, the PRL-induced increase in Sca1 was abolished in NIH3T3 cells (**Figure 3D-F**). The results indicate that the activation of STAT3 is required in the PRL-induced Sca1 expression in NIH3T3 cells.

PRL induces development of fibrofatty cells

The results of **Figures 1-4** implicate that PRL may induce fibroblasts to fibrofatty cells in the heart. To test the inference, we added PRL to the NIH3T3 cell culture. Four weeks later, the cells show apparent collagen expression (**Figure 4A**), indicating that, after exposing to PRL in the culture for 4 weeks, the cells still keep the feature of fibrocytes. The cells also showed high levels of adiponectin, fatty acid binding protein (aP2), peroxisome proliferator activated receptor-g (PPARg) and CCAAT/enhancer binding protein (C/EBP)α (**Figure 4B-E**), indicating that these cells have gained the feature of adipocytes. On the other hand, after culturing with medium alone for 4 weeks, the control NIH3T3 cells only show high levels of collagen (**Figure 4F**); the adipocyte-related molecules were barely detectable in these cells (**Figure 4G-J**), indicating that fibroblasts do not

gain the features of adipocytes automatically. The results of flow cytometry (**Figure 4A-J**) were supported by the data of RT-qPCR (**Figure 4K**) and Western blotting (**Figure 4L**) respectively. The results suggest that PRL induces the development of fibrofatty cells from fibroblasts.

Discussion

ARVC is one of the diseases causing sudden death. There are no specific remedies for the treatment of ARVC. The specific diagnostic parameters are also lacking. Thus, it is of significance to understand the pathogenesis of ARVC. The present data show that, in an animal model study, psychological stress induces fibrofatty cells in the mouse heart, which is mediated by PRL. The data are supported by the data of cell culture study. After culturing with PRL, the NIH3T3 cells still retain the feature of fibrocytes (expressing collagen), and have gained the features of adipocytes (expressing adiponectin, aP2, PPARg and C/EBPa), which suggests that PRL induces the development of fibrofatty cells from fibroblasts. The data suggest that psychological stress-released PRL is capable of inducing the fibrofatty cells in the heart. Administration with antagonists of PRL prevents the induction of the fibrofatty cells in the heart. In addition, we also identified a large number of fibrofatty cells in 18/32 ARVC patients.

It is well documented that PRL plays a critical role in the psychological stress-induced pathological lesions in the body. Yang et al. indicate that PRL mediates the stress-induced intestinal epithelial barrier dysfunction [25]. Akiyama et al. indicate that PRL mediates the stress-induced tracheal epithelial barrier dysfunction [26]. Prolactin also plays an important role in the pathogenesis of cardiac dysfunction in patients with peripartum cardiomyopathy [27]. It is also suggested that exposure to psychological stress is a risk factor for cardiovascular disease; it may play a role in the development of cardiovascular pathology [28]. Our data are in line with those studies by showing that the PRL can mediate the effect of psychological stress on inducing the fibrofattry cells in the mouse heart.

PRL has two receptors, PRL-R1 and PRL-R2. Our data indicate that the mouse cardiac tissue has both PRLR1 and PRLR2. A number of published studies indicate the heart tissue expresses PRL-R [29]. Our data are similar to the previous reports by showing low levels of PRLR1 and PRLR2, which were markedly increased in the heart tissue after stress treatment. The fact suggests that the chronic stress can up regulate the expression of PRLR in the heart. Thus, administration with PRLR antagonists is an option to block the effect of PRL on the heart.

Accumulation of fat laden cells in the ventricle is a major morphologic hallmark of ARVC; these fat laden cells are designated fibrofattry cells. In fact, a certain amount of fibrofatty cell is present in the ventricular and apical regions in the normal heart [30]. Our data also show a small amount fibrofatty cell in the normal control and sham stress control mice. The $Sca1^+$ cells have the tendency to differentiate into adipocytes [31]. In our study, although the expression is trifle in the normal ventricular tissue, the expression of Sca1 is significantly increased in the heart tissue after stress treatment. The data are supported by the cell culture experiments, in which PRL markedly up regulates the expression of Sca1 in NIH3T3 cells, a fibroblast cell line. PRL is one of the major psychological stress-related mediators; it has multiple functions. Our study has revealed a novel aspect of this stress-mediator by showing that PRL can modulate the expression of Sca1 via regulating the STAT3 phosphorylation. Others also suggest that STAT3 is involved in the adipocyte differentiation [32].

In the presence of PRL, the NIH3T3 cells differentiated into adipocyte-like cells. These cells also express collagen. The fact indicates that PRL induces the fibroblast to differentiate into fibrofatty cells. The data also show that after treatment with PRL, the cells express adiponectin, aP2, PPARg and C/EBPa. These molecules can be secreted by adipocytes [33, 34]. Our data show that, after treatment with PRL, the NIH3T3 cells express high levels of adiponectin, aP2, PPARg and C/EBPa, indicating these cells have the features of adipocytes.

In summary, the present data show that the stress-derived PRL can induce fibrofatty cells in the mouse heart, which can be blocked by administration with PRL antagonists during the stress period.

Acknowledgements

This study was supported by the Chinese government "863" program "Key technology of heart transplantation and immune regulation" (NO: 2012AA021009. Grantees: Dr. Hu and Dr. Song).

Disclosure of conflict of interest

None.

Authors' contribution

MW, XC, LL, LC, ZS, XT, YX, KC, KZ and HJ performed the experiments, analyzed data and reviewed the manuscript. JS and HJF designed the project, supervised the experiments and wrote the paper.

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