Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.jzus.zju.edu.cn; www.springerlink.com
E-mail: jzus@zju.edu.cn

PGC-1α coordinates with Bcl-2 to control the cell cycle in U251 cells through reducing ROS*#

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Received Mar. 15, 2017; Revision accepted June 12, 2017; Crosschecked May 14, 2018

Abstract: B-cell lymphoma 2 (*Bcl-2*) has a dual function, acting as both an oncogene and an anti-tumor gene. It is well known that Bcl-2 exerts its tumor promoting function through the mitochondrial pathway. However, the mechanism by which it suppresses tumor formation is not well understood. We have previously shown that Bcl-2 inhibits cell cycle progression from the G_0/G_1 to the S phase after serum starvation, and that quiescent Bcl-2 expressing cells maintain a significantly lower level of mitochondrial reactive oxygen species (ROS) than control cells. Based on the fact that ROS mediate cell cycle progression and are controlled by peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α), a key molecule induced by prolonged starvation and involved in mitochondrial metabolism, we hypothesized that PGC-1α might be related to the cell cycle function of Bcl-2. In this paper, we show that PGC-1α is upregulated by Bcl-2 overexpression and downregulated following Bcl-2 knockdown or downregulation after serum starvation. However, Bcl-2 is negatively regulated by PGC-1α expression. Further, co-immunoprecipitation (co-IP) experiments showed that PGC-1α protein is co-precipitated with Bcl-2 at the G_0/G_1 phase. Taken together, our results suggest that PGC-1α interacts with Bcl-2 after serum depletion, and that Bcl-2 might recruit PGC-1α to reduce ROS, which in turn delays cell cycle progression in coordination with Bcl-2.

Key words: B-cell lymphoma 2 (Bcl-2); Peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α); Mitochondria; Reactive oxygen species (ROS); Cell cycle
https://doi.org/10.1631/jzus.B1700148 **CLC number:** Q25

1 Introduction

B-cell lymphoma 2 (*Bcl-2*) has both pro- and anti-apoptotic potential. It is well known that the Bcl-2 protein is anchored to the mitochondrial outer membrane, and antagonizes the pro-apoptotic protein Bax (Bcl-2-associated X protein) by forming Bcl-2/Bax heterodimers that control mitochondrial membrane permeability and promote tumorigenesis (Hockenbery et al., 1990). Bcl-2 acts as an anti-tumor gene in early stage solid tumors, where it significantly reduces tumor incidence by regulating the cell cycle (Murphy et al., 1999; Vail et al., 2001). In vitro cell studies have demonstrated that the most pronounced cell cycle effect of Bcl-2 is to delay the progression from G_0/G_1 to S phase (Janumyan et al., 2003, 2008). This has been confirmed in a Bcl-2 transgenic mouse model, where proliferation of lymphoid T cells was restrained and tumor-associated morbidity was

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* Project supported by the National Natural Science Foundation of China (Nos. 81760507 and 81360310) and the Yunnan Province Science and Technology Innovation Team (No. 2011CI123), China
# Electronic supplementary materials: The online version of this article (https://doi.org/10.1631/jzus.B1700148) contains supplementary materials, which are available to authorized users
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significantly decreased (Cheng et al., 2004). However, the signaling pathways by which Bcl-2 suppresses tumor formation are not fully understood.

We have recently observed that quiescent Bcl-2 overexpressing cells maintain less mitochondrial reactive oxygen species (ROS) than control cells: elevation of ROS could accelerate cell cycle progression (Du et al., 2017). These findings support the hypothesis that mitochondrial oxidative phosphorylation (OXPHOS) plays a key role in the anti-proliferation function of Bcl-2. The most important factor identified to modulate mitochondrial biogenesis and bioenergetics is peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α), which is predominantly expressed in tissues with high energy demands, such as the heart, brain, and muscle (Wu et al., 1999; McBride et al., 2006; Ventura-Clapier et al., 2008). As a co-activator, PGC-1α interacts with a broad range of transcription factors and plays an important role in glucose metabolism and prolonged starvation (Meirhaeghe et al., 2003). PGC-1α can translocate from the nucleus into the mitochondrial displacement loop (D-loop) region, where it forms a complex with mitochondrial transcription factor A in an adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK)-dependent manner (Aquilano et al., 2010; Safdar et al., 2011; Smith et al., 2013). We recently found that PGC-1α regulates the cell cycle through adenosine triphosphate (ATP) and ROS signals in fibroblast CH1 cells (Fu et al., 2016). Therefore, we hypothesized that Bcl-2 recruits PGC-1α into mitochondria to coordinate tumor suppression functions.

In this study, we investigated whether Bcl-2 interacts with PGC-1α to delay entry from the G_0/G_1 to the S phase of the cell cycle by synchronizing and analyzing cells in the G_0/G_1 phase under serum starvation (SS) or contact inhibition (CI). Human glioma U251 cells with high expression of endogenous PGC-1α were chosen to study the relationship between Bcl-2 and PGC-1α. We also evaluated this relationship using co-immunoprecipitation (co-IP).

2 Materials and methods

2.1 Cell lines and cell culture

Human kidney epithelial 293T cells and human glioma U251 cells (with high Bcl-2 and PGC-1α endogenous expression) were supplied by the Biochemistry and Molecular Biology Laboratory of Yunnan University, China. Mouse embryonic fibroblasts NIH3T3 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Logan, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; BI, Logan, USA) and 1% (v/v) penicillin/streptomycin (Hyclone, Logan, USA) at 37 °C in 5% CO_2.

2.2 Lentivirus packaging and stable cell transfection

Recombinant pBABEpuro-*Bcl-2* vector, packaging plasmid pCLECO, and X-tremeGENE HP DNA transfection reagent (Roche, Basel, Switzerland) were added in DMEM, mixed gently, and incubated at room temperature for 20 min. The mixture was added drop-wise into 293T cells in a 10-cm plate. After 48 h, the supernatant containing pBABEpuro-Bcl-2 virus was collected and filtered through a 0.45-μm filter. NIH3T3 cells were then infected with the virus. After 48 h of culture, the aminoglycoside antibiotic puromycin (Gibco-BRL, Carlsbad, USA) was added into the medium at a final concentration of 4 μg/ml to select NIH3T3 cells with stable expression of Bcl-2. Bcl-2 expression was confirmed by Western blotting (Fu et al., 2016).

2.3 Cell cycle synchronization and analysis by flow cytometry

For cell synchronization via SS, U251 or NIH3T3 cells were washed three times with phosphate-buffered saline (PBS) and cultured for 48 h in medium containing no FBS (U251 cells) or containing 0.1% (v/v) calf serum (NIH3T3 cells). For cell synchronization via CI, the cells were allowed to reach confluence and then maintained in culture for 5 d. Synchronized cells by either method were harvested, washed with cold PBS, and incubated with a solution containing 50 μg/ml propidium iodide (PI) and 0.03% (v/v) Triton X-100 at room temperature for 20 min. For each sample, at least 2×10^5 cells/ml were analyzed with a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA). Cell cycle profiles were calculated using the C6 software (Fu et al., 2016).

2.4 Western blotting

Cells were harvested, washed twice with PBS, incubated in radioimmunoprecipitation assay lysis buffer (Beyotime, Jiangsu, China) on ice for 20 min,

and centrifuged at 10000*g* for 15 min at 4 °C. The supernatant was collected and protein concentration was quantified with a bicinchoninic acid (BCA) protein assay kit (Dingguo, Beijing, China). Supernatant samples (50 μg proteins) were loaded onto a 12.5% (0.125 g/ml) polyacrylamide gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene fluoride (PVDF) membrane at constant voltage (100 V) for 2 h. The membrane was then blocked with 5% (0.05 g/ml) milk and probed with primary antibody (at 1:1000 dilution) overnight at 4 °C. After being washed three times with PBST (PBS with Triton X-100), the membrane was incubated with a secondary antibody (at 1:2000 dilution) at room temperature for 2 h, and the signal was developed with an enhanced chemiluminescence kit (Thermoscientific, Boston, USA). The quantification of relative protein expression based on Western blotting signals was performed using the ImageJ software. Antibodies against PGC-1α and p27 were purchased from the Cell Signaling Technology Co. (CST, Boston, USA), anti-tubulin antibody from the Beyotime Company (Jiangsu, China), and anti-Bcl-2 antibody from Becton, Dickinson and Company (BD, USA). Light chain-specific horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody was purchased from Jackson Immunoresearch Laboratories Inc. (Jackson, USA).

2.5 Gene knockdown by siRNA

Small interfering RNAs (siRNAs) for human *PGC-1α* (sense: 5'-GUCGCAGUCACAACACUUATT-3', antisense: 5'-UAAGUGUUGUGACUGCGACTT-3'), control (sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3'), and human *Bcl-2* (*Bcl-2*-homo-506 sense: 5'-GGGAGAACAGGGUACGAUATT-3', antisense: 5'-UAUCGUACCCUGUUCUCCCTT-3'; *Bcl-2*-homo-528 sense: 5'-GGGAGAUAGUGAUGAAGUATT-3', antisense: 5'-UACUUCAUCACUAUCUCCCTT-3'; *Bcl-2*-homo-928 sense: 5'-GAGGAUUGUGGCCUUCUUUTT-3', antisense: 5'-AAAGAAGGCCACAAUCCUCTT-3') were purchased from GenePharma (Shanghai, China). Transfections were performed according to the X-tremeGENE HP Transfection Reagent's protocol. Briefly, 160 pmol siRNA and 10 μl X-tremeGENE HP transfection reagent were diluted in 50 μl DMEM, respectively, incubated for 5 min, gently mixed together, and incubated for 15 min at room temperature to form siRNA-X-tremeGENE HP complexes, which were then added to 7×10^5 cells per well plated in a 6-well dish and incubated for 48 h (Fu et al., 2016).

2.6 Co-immunoprecipitation assay

Co-IP experiments were performed using the Pierce Classic IP kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Cell lysate supernatants were probed with an anti-Bcl-2 antibody. Light chain-specific HRP-conjugated anti-rabbit IgG secondary antibody was used to eliminate heavy chain interference.

2.7 Immunofluorescence staining

3T3PB and 3T3Bcl-2 cells were seeded in a 12-well plate with DMEM (containing 10% (v/v) FBS) at 37 °C in 5% CO_2. After 24 h, cells were fixed with 4% methanol for 10 min, washed twice with PBS, and incubated with Bcl-2 and PGC-1α antibodies (at 1:100 dilution) for 2 h at 37 °C, then stained with CY3 antibodies or fluorescein isothiocyanate (FITC)-labeled (at 1:200 dilution) for 1 h. Immunofluorescence images were taken under the confocal microscope (Leica, Berlin, Germany).

3 Results

3.1 PGC-1α expression increased in Bcl-2 over-expressing cells

Mouse embryonic fibroblast NIH3T3 cells were transfected with *Bcl-2* cloned in a pBABEpuro plasmid (referred to as 3T3Bcl-2 in this paper). Cells transfected with the empty vector served as the control (referred to as 3T3PB in this paper). We have previously shown that Bcl-2 and PGC-1α regulate the cell cycle, and Bcl-2 functions during the G_0/G_1 stage (Janumyan et al., 2008; Fu et al., 2016; Du et al., 2017). Therefore, we compared PGC-1α expression between 3T3Bcl-2 and 3T3PB, which were synchronized at the G_0/G_1 stage by SS and CI. Both 3T3PB and 3T3Bcl-2 were arrested successfully in the G_0/G_1 phase after SS, and we found that the ratio of 3T3PB cells in the S phase dropped from (19.4±1.1)% (normally growing, NG3T3PB) to (2.8±0.1)% (serum-starved, SS3T3PB), and the ratio of 3T3Bcl-2 in the S phase dropped from (18.6±0.9)% (normally growing, NG3T3Bcl-2) to (3.0±0.2)% (serum-starved,

SS3T3Bcl-2) (Figs. 1a and 1b). We observed a significant elevation in p27 levels in SS3T3Bcl-2 cells, confirming that Bcl-2 has an anti-apoptotic function (Figs. 1c and 1d). As we expected, PGC-1α expression was clearly higher in SS3T3Bcl-2 than in NG3T3Bcl-2 cells, while no significant difference was noticed between SS3T3PB and NG3T3PB cells (Figs. 1c and 1e). Together with our previous report (Fu et al., 2016) that PGC-1α reduced ROS, this result suggests that increased PGC-1α might assist Bcl-2 cells to reduce ROS, which in turn delays S phase re-entry after prolonged SS.

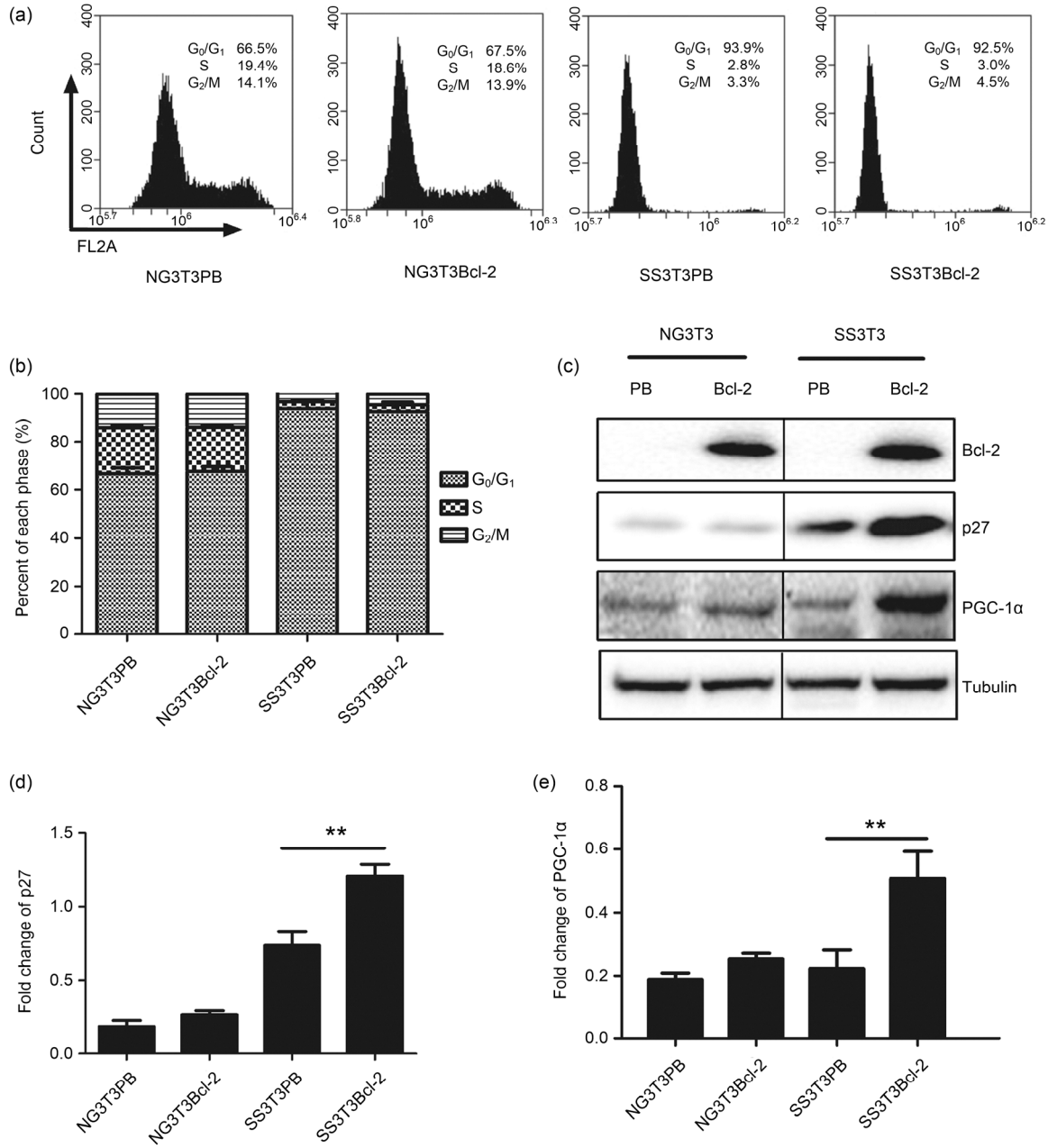

Fig. 1 Comparison of p27 and PGC-1α expression between SS-treated and NG 3T3 cells

(a) 3T3PB (pBABEpuro, empty vector) or 3T3Bcl-2 (pBABEpuro-Bcl-2) cells were cultured for 48 h in 0.1% (v/v) serum and harvested for cell cycle analysis by flow cytometry. (b) The percentage of SS-treated or NG 3T3 cells in each cell cycle phase. (c) Expression of PGC-1α and p27 in 3T3 cells was measured by Western blotting. (d, e) Quantifications of PGC-1α and p27 protein expression shown in (c). Data are expressed as mean±standard deviation (n=3). ** P<0.01. NG: normally growing; SS: serum starvation

To avoid experimental bias, the results were verified in cells arrested by CI. Consistent with the results from the SS method (Fig. 1), a significant ratio of both 3T3PB and 3T3Bcl-2 cells was arrested in the G_0/G_1 phase (CI3T3PB and CI3T3Bcl-2, respectively; Figs. 2a and 2b). We also found clearly increased PGC-1α and p27 expression in CI3T3Bcl-2 compared with both NG3T3Bcl-2 and CI3T3PB cells, whereas PGC-1α expression did not significantly change between NG3T3PB and CI3T3PB cells (Figs. 2c–2e). Based on the combined results from SS (Fig. 1) and CI (Fig. 2) treatments, we conclude that during the G_0/G_1 phase, Bcl-2 might recruit PGC-1α to block cell cycle progress.

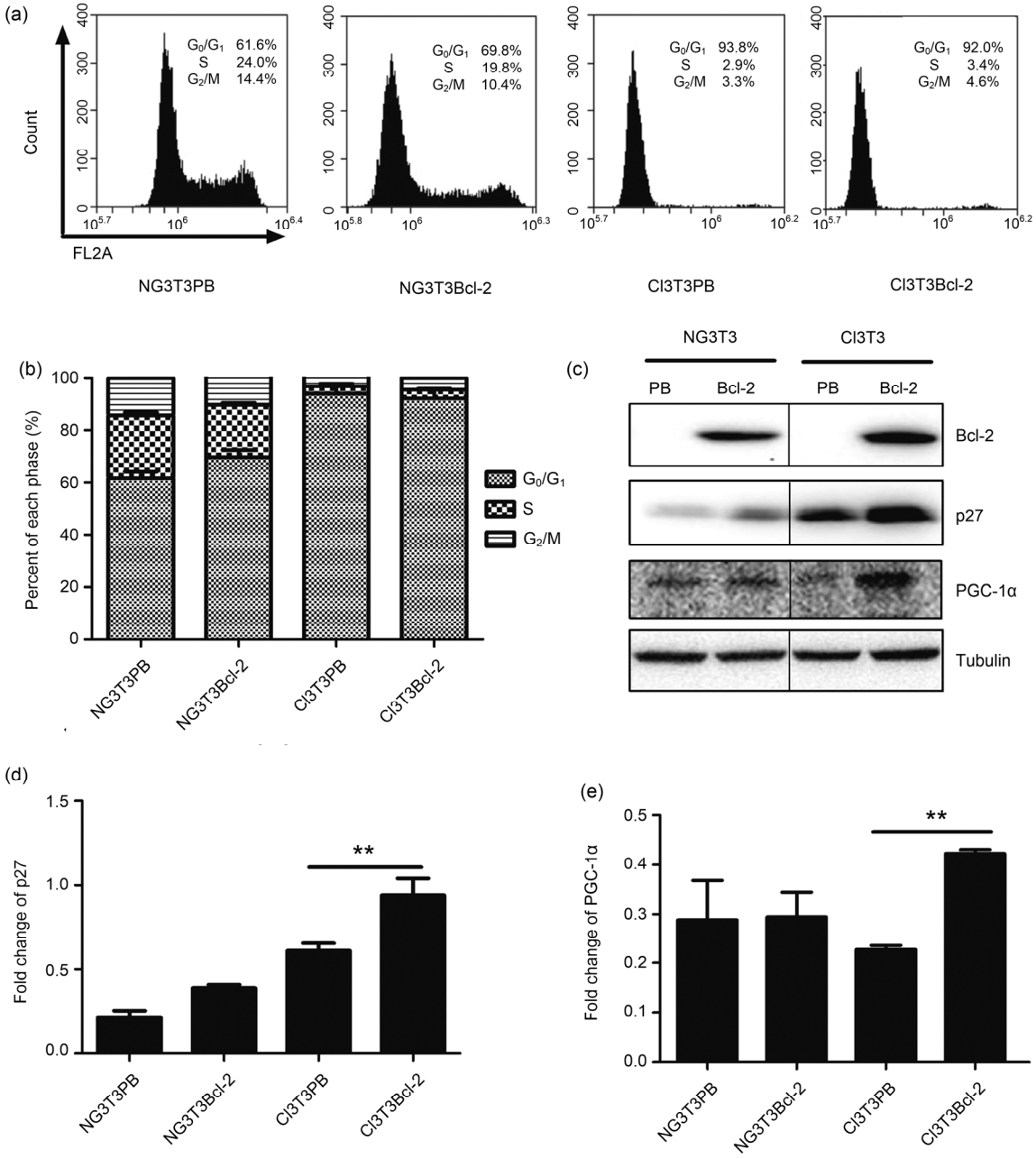

Fig. 2 Comparison of p27 and PGC-1α expression between CI-treated and NG 3T3 cells

(a) 3T3PB (pBABEpuro, empty vector) or 3T3Bcl-2 (pBABEpuro-Bcl-2) cells were contact-inhibited for 72 h and harvested for cell cycle analysis by flow cytometry. (b) The percentage of CI-treated or NG 3T3 cells in each cell cycle phase. (c) Expression of PGC-1α and p27 in 3T3 cells was measured by Western blotting. (d, e) Quantifications of PGC-1α and p27 protein expression shown in (c). Data are expressed as mean±standard deviation (n=3). $^{**}P<0.01$. NG: normally growing; CI: contact inhibition

3.2 PGC-1α decreased after Bcl-2 knockdown

Since PGC-1α was upregulated in Bcl-2 over-expressing cells (Figs. 1 and 2), we further investigated the effects of Bcl-2 knockdown on PGC-1α in glioma U251 cells, which have high Bcl-2 and PGC-1α endogenous expression. Three specific siRNAs (506, 528, and 928) were used to interfere with Bcl-2 expression, while a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA and a mimic siRNA were used as positive and negative controls, respectively (Fig. 3a). Western blotting analysis confirmed successful Bcl-2 knockdown by all three specific siRNAs, while Bcl-2 expression was not affected in either the positive or negative control (Fig. 3b). Interestingly, PGC-1α expression was decreased significantly in cells transfected with siRNAs 528 and 928 targeting Bcl-2, but not in the control cells (Fig. 3c). Bcl-2-specific siRNA 506 showed less significant effects on Bcl-2 expression than did siRNAs 528 and 928 (Fig. 3b). Similarly, Western blotting analysis demonstrated that PGC-1α expression decreased less in cells transfected with siRNA 506 than in those transfected with 528 or 928 (Fig. 3c), suggesting a dose-dependent interfering effect. Thus, these results support the hypothesis that PGC-1α expression correlates with Bcl-2 expression.

3.3 Bcl-2 upregulation correlates with PGC-1α downregulation in U251 cells after SS treatment

Next, we investigated whether Bcl-2 was regulated by PGC-1α. Since PGC-1α and Bcl-2 endogenous expression is high in the human glioma cell line U251, we chose these cells to knockdown PGC-1α and checked the effects on Bcl-2. U251 cells were treated by SS for 24, 48, and 72 h, and normally growing (NGU251) cells were used as a control. The results showed that (19.7±1.1)% of NGU251 cells were in the S phase, whereas after 24, 48, and 72 h of SS treatment (6.7±0.5)%, (2.6±0.2)%, and (2.3±0.2)% of cells were found in the S phase, respectively (Figs. 4a and 4b). Western blotting analysis showed that after prolonged SS, Bcl-2 was upregulated following PGC-1α downregulation (Fig. 4c). These results demonstrate that Bcl-2 responds to PGC-1α in a negative way.

3.4 Bcl-2 was upregulated following PGC-1α knockdown

To further test the relationship between PGC-1α and Bcl-2, experiments with siRNA targeting PGC-1α were performed. Fig. 5a shows that PGC-1α was significantly decreased in cells transfected with siRNA of PGC-1α, while no change was found in cells transfected with the negative control, positive control (GAPDH) or the transfection reagent only (Fig. 5b), indicating successful PGC-1α knockdown by siRNA. Western blotting analysis showed that Bcl-2 expression was increased in cells transfected with siRNA targeting PGC-1α, compared with the control or otherwise treated cells (Fig. 5c). Based on these results and those shown in Fig. 4, we conclude that Bcl-2 is negatively regulated by PGC-1α.

3.5 Bcl-2 might coordinate with PGC-1α in U251 cells

These results reveal that Bcl-2 correlates with PGC-1α, and we hypothesize that Bcl-2 recruits PGC-1α to the mitochondrial membrane, and directly interacts with the Bcl-2 protein. We tested this hypothesis in the co-IP experiment.

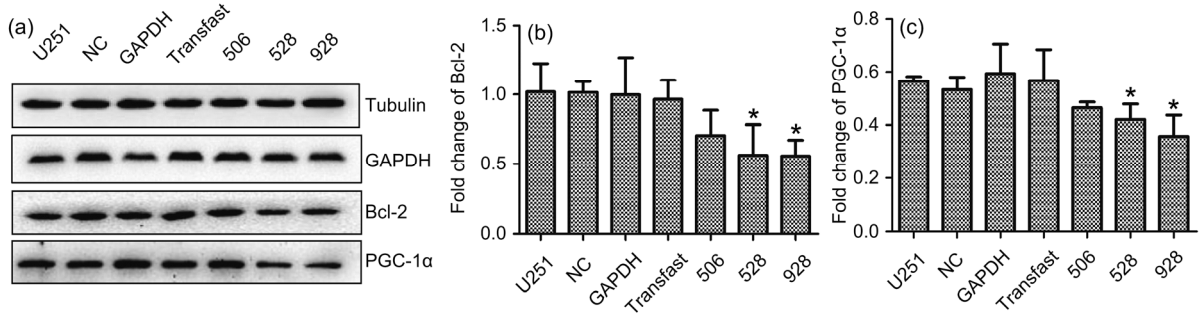

Fig. 3 Bcl-2 and PGC-1α knockdown by Bcl-2 siRNAs in U251 cells

(a) GAPDH, Bcl-2, and PGC-1α expression was detected by Western blotting after transfection with Bcl-2-specific siRNAs (506, 528, and 928). (b, c) Quantifications of Bcl-2 and PGC-1α protein expression shown in (a). Data are expressed as mean±standard deviation (n=3). * P<0.05, vs. tubulin. NC: random siRNA serving as a negative control; GAPDH: positive control; Transfast: transfection reagents only

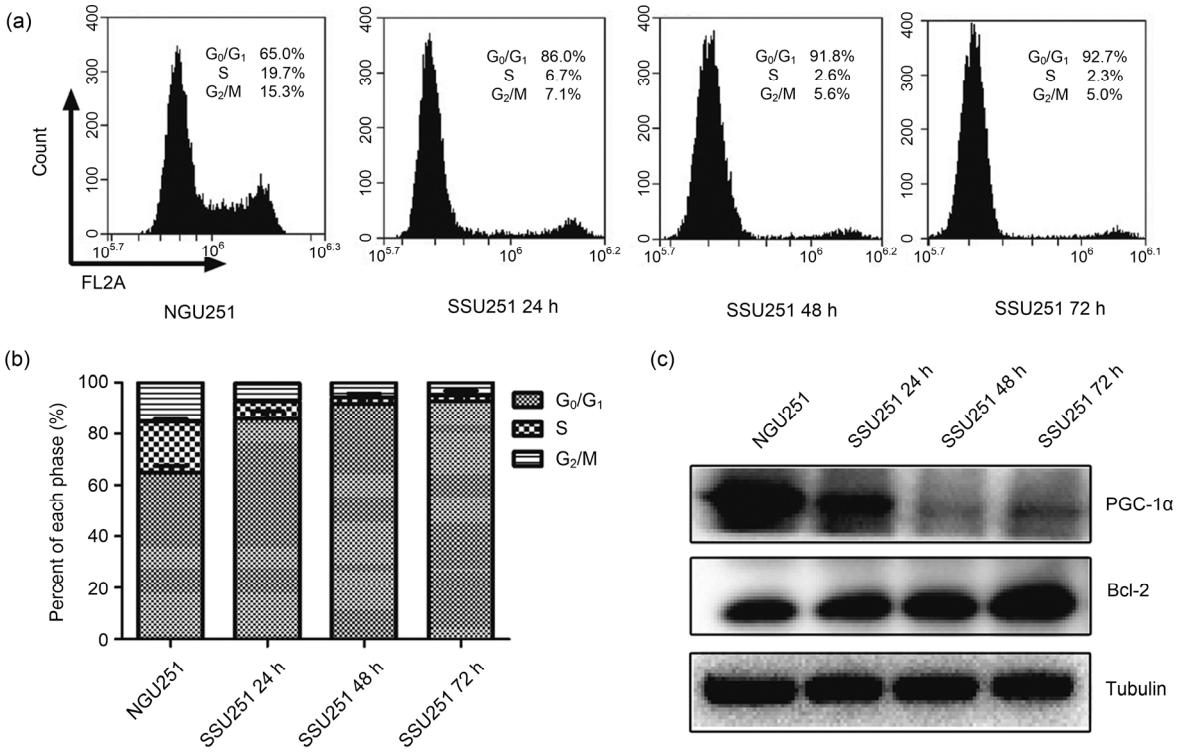

Fig. 4 Cell cycle profiles and Bcl-2 and PGC-1α expression after SS treatment of U251 cells

(a) U251 cells were cultured in serum-free medium for 24, 48, and 72 h, and harvested for cell cycle analysis by flow cytometry. (b) The percentage of SS-treated or NG U251 cells in each cell cycle phase. Data are expressed as mean±standard deviation (n=3). (c) PGC-1α and Bcl-2 expression in U251 cells after SS treatment was measured by Western blotting

Serum-starved U251 or NIH3T3 cells were harvested and lysed with an immunoprecipitation buffer. PGC-1α was pulled down by an anti-Bcl-2 antibody and analyzed by Western blotting. A significant band was detected in the pull-down lane after probing with an anti-PGC-1α antibody, but not in the IgG lane (Fig. 6). These results indicate that Bcl-2 directly binds to PGC-1α during SS.

4 Discussion

The human *Bcl-2* gene was first identified in tumor cells of follicular lymphoma patients, and was localized near the junction, at which chromosomes 18 and 14 (t(14;18)) are joined (Tsujimoto et al., 1984). This chromosome translocation led to upregulation of Bcl-2 expression and contributed to cancer (Tsujimoto et al., 1985; Nunez et al., 1989). As an oncogene, Bcl-2 can inhibit apoptosis and promote cell survival, and these functions have been well characterized for decades. Proapoptotic Bax was first identified as an inhibitory binding partner of Bcl-2 (Oltval et al., 1993). Bax is activated by a range of cell death signals, which cause conformational changes and oligomerization to form a channel, through which cytochrome *c* exits from mitochondria to trigger caspase activation and cell death (Hardwick and Soane, 2013). Bcl-2 could bind to proapoptotic partners to form heterodimers that block cell death. The balance between anti- and pro-apoptotic functions of the Bcl-2 family of proteins ultimately determines cell fate (Korsmeyer et al., 1993). However, the anti-tumor function of Bcl-2 is still not fully understood. Our group discovered that Bcl-2 could retard cell cycle progression through inhibiting p27 protein degradation (Janumyan et al., 2008; Du et al., 2017), but further investigation demonstrated that no direct interaction existed between Bcl-2 and p27 (data not shown).

Our recent work indicated that when cells are synchronized at the G_0/G_1 phase by SS, Bcl-2 overexpressing cells maintain a significantly lower ROS

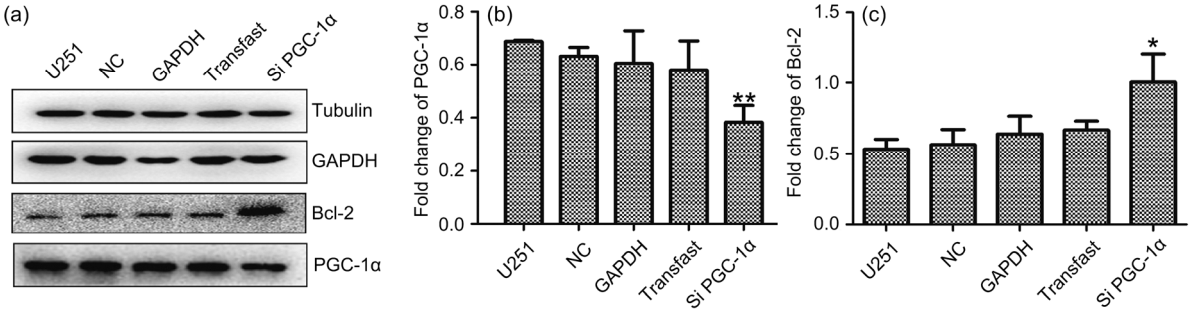

Fig. 5 Upregulated Bcl-2 following PGC-1α knockdown by PGC-1α siRNA in U251 cells

(a) GAPDH, Bcl-2, and PGC-1α expression was measured by Western blotting after transfection with PGC-1α-specific siRNA. (b, c) Quantifications of Bcl-2 and PGC-1α protein expression shown in (a). Data are expressed as mean±standard deviation (n=3). * P<0.05, ** P<0.01, vs. tubulin. NC: random siRNA serving as a negative control; GAPDH: positive control; Transfast: transfection reagents only; Si PGC-1α: PGC-1α-specific siRNA

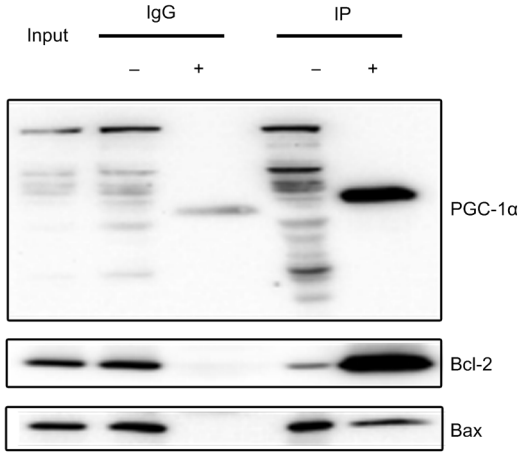

Fig. 6 PGC-1α and Bcl-2 co-IP in SSU251 cells

PGC-1α was pulled down by anti-Bcl-2 antibody, as well Bcl-2 and Bax were analyzed by Western blotting. Input: protein lysate before co-IP; IgG: isotype control with co-IP antibody; IP: conjunct with anti-Bcl-2 antibody

level compared with control cells. However, there were no differences in ROS levels between normally growing Bcl-2 overexpressing cells and control cells. This is consistent with the fact that Bcl-2's anti-tumor function is activated only in the G_0/G_1 stage but not during normal growth (Janumyan et al., 2008). These results indicate strongly that ROS play a role in Bcl-2's cell cycle function (Du et al., 2017). Mitochondrial OXPHOS impinges on many cellular functions, including energy allocation and programmed cell death. ROS generated by mitochondrial OXPHOS provide a signaling system from mitochondria to the nucleus (Hansen et al., 2006) and are involved in cell cycle regulation, with low levels of ROS suppressing cellular growth and proliferation (Burdon, 1995; Heiden et al., 2009). Bcl-2 was reported to have anti-oxidant effects as an ROS scavenger, but it lacks the sequence or structural features of known anti-oxidant proteins, suggesting that Bcl-2 might clear ROS by modulating mitochondrial bioenergetics (Susnow et al., 2009).

PGC-1α is a transcriptional co-activator of peroxisome proliferator-activated receptor γ (PPARγ) that regulates transcription factor activity, and has a central place in multiple cellular processes, including mitochondrial respiration and OXPHOS (Bagattin et al., 2010). PGC-1α responds to metabolic challenges such as exercise, starvation, or cold; its expression is induced by these environmental stimuli and is associated with cancer (Yoon et al., 2001). We previously reported that as a master modulator of mitochondrial OXPHOS, PGC-1α reduced the ROS level to regulate the cell cycle (Fu et al., 2016). These findings led to the hypothesis that PGC-1α might directly or indirectly coordinate with Bcl-2 in regulating the cell cycle. To verify this hypothesis, we experimented with embryonic fibroblast NIH3T3 cells, in which Bcl-2 was either overexpressed or silenced, and which were arrested by SS or CI to activate the cell cycle function of Bcl-2. We observed that during SS, the expression of p27 was increased significantly in G_0/G_1-arrested Bcl-2 overexpressing cells compared with the control cells, which validated the cell cycle function of Bcl-2 (Janumyan et al., 2003, 2008; Cheng et al., 2004). PGC-1α was also elevated in arrested Bcl-2 overexpressing cells but not in control cells (Figs. 1 and 2), suggesting a relationship between PGC-1α and Bcl-2. We hypothesized that Bcl-2 recruits and relies on PGC-1α to reduce ROS,

which is a critical signal in mediating the cell cycle. To test this possibility, Bcl-2 knockdown by siRNA was performed, and as we expected, PGC-1α expression was correspondingly decreased (Fig. 3). Given that PGC-1α regulates mitochondrial function to eliminate ROS and arrested Bcl-2 cells contain less ROS, these results suggest that Bcl-2 recruits PGC-1α to reduce ROS levels and block cell cycle progression.

To further clarify the relationship between Bcl-2 and PGC-1α, we investigated the influence of PGC-1α knockdown on Bcl-2 expression. Endogenous expression of PGC-1α is low in normally growing 3T3Bcl-2 and control cells (Figs. 1c and 2c). We failed to overexpress PGC-1α in Bcl-2 cells for unknown reasons and thus chose human glioma U251 cells with high expression of endogenous PGC-1α to perform the experiments. Fig. 5 shows that Bcl-2 expression was upregulated after PGC-1α was successfully silenced. Consistent with this result, Bcl-2 was upregulated following PGC-1α downregulation during SS treatment (Fig. 4). These results suggest a negative feedback between Bcl-2 and PGC-1α expression.

Our findings indicate that PGC-1α interacts with Bcl-2 directly or indirectly to regulate cell cycle progression. In our co-IP experiment, after probing with an anti-PGC-1α antibody (Fig. 6), a significant band was shown at the Bcl-2 pull-down group, which indicates the direct interaction between PGC-1α and the Bcl-2 protein. The immunofluorescence images also showed the co-localization of PGC-1α and Bcl-2 proteins in the cytoplasm of NIH3T3 cells; however, their subcellular localization needs further study (Fig. S1).

Many studies have confirmed that ATP and ROS are key signals in regulating the cell cycle. Although it is known that Bcl-2 is involved in regulating the cell cycle via the ROS pathway, its mechanism of action remains unclear. Bcl-2 lacks the sequence or structural features of known anti-oxidant proteins (Susnow et al., 2009), suggesting that it controls ROS indirectly via other factors. PGC-1α was identified as a crucial switch on mitochondrial OXPHOS (Bagattin et al., 2010), which functions to diminish ROS (Fu et al., 2016). Based on our current study, we conclude that when tumor cells grow rapidly and start confronting SS, PGC-1α binds directly to overexpressed Bcl-2, reducing ROS to inhibit the cell cycle and suppress tumor cell proliferation.

Contributors

Kun YAO and Xu-feng FU conceived, designed, and performed the experiments, wrote the paper, and prepared figures. Xing DU, Yan LI, and Shan-shan YANG analyzed the data and contributed reagents/materials. Min YU reviewed drafts of the paper. Qing-hua CUI conceived and designed the experiments, wrote the paper, and reviewed drafts of the paper.

Acknowledgements

We would like to thank all the members of the Key Laboratory for Biochemistry and Molecular Biology of High Education in Yunnan Province, Kunming, China, for their participation in this study.

Compliance with ethics guidelines

Kun YAO, Xu-feng FU, Xing DU, Yan LI, Shan-shan YANG, Min YU, and Qing-hua CUI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Fig. S1 Immunofluorescence images of PGC-1α and Bcl-2 protein co-localization in NIH3T3 cells

中文概要

题　目：PGC-1α 在 U251 细胞中协同 Bcl-2 通过降低 ROS 来调控细胞周期

目　的：探究过氧化物酶体增生激活受体 γ 协同刺激因子 1α （PGC-1α）和 B 细胞淋巴瘤-2（Bcl-2）在调控细胞周期中的相互关系。

创新点：首次证明在血清饥饿时 PGC-1α 负调控 Bcl-2，并且认为 Bcl-2 可能通过招募 PGC-1α 降低细胞中的活性氧自由基（ROS）以调节细胞周期。

方　法：用蛋白质印迹法（Western blotting）检测了接触抑制和血清饥饿处理的 NIH3T3 过表达 Bcl-2 的细胞中 PGC-1α 的表达，并且分别检测了用 Bcl-2 和 PGC-1α 的小干扰 RNA（siRNA）降低 U251 细胞（内源性高表达 Bcl-2 和 PGC-1α）中的 Bcl-2 和 PGC-1α 的表达，最后用免疫共沉淀（co-IP）检测了二者的关系。

结　论：本实验中用两种细胞同步化的方法（接触抑制和血清饥饿）处理了 Bcl-2 过表达的 NIH3T3 细胞时发现 PGC-1α 高表达，用 Bcl-2 的 siRNA 处理了 U251 细胞时发现 PGC-1α 的表达降低，但是血清饥饿处理了 U251 后发现 Bcl-2 升高而 PGC-1α 降低，而且 PGC-1α 被 siRNA 降低后 Bcl-2 反而上升，最后用 Bcl-2 抗体免疫共沉淀了 PGC-1α 蛋白，这些结果说明在血清饥饿时 PGC-1α 负调控 Bcl-2 行使调节细胞周期的功能。

关键词：过氧化物酶体增生激活受体γ协同刺激因子1α（PGC-1α）；B细胞淋巴瘤-2（Bcl-2）；线粒体；活性氧自由基（ROS）；细胞周期