Running title: PGC-1α inhibits breast cancer proliferation via SIRT3

PGC-1α activates SIRT3 to modulate cell proliferation and glycolytic metabolism in breast cancer

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Breast cancer is the leading cause of death among women. PGC-1α plays an important role in the regulation of metabolic reprogramming in cancer cells. SIRT3 has significant implications for tumor growth. In this study, we explored the roles of PGC-1α and SIRT3 in cell proliferation and mitochondrial energy metabolism alterations in breast cancer cells. The expression patterns of PGC-1α and SIRT3 were examined using qRT-PCR and western blotting analysis. MCF-7 and MDA-MB-231 cells were infected by adenovirus to overexpress or knock down the expression of PGC-1α and SIRT3. Cell viability and apoptosis were analyzed by CCK-8 and flow cytometry, respectively. Hexokinase2, pyruvate kinase activities, as well as NAD+/NADH ratio and ATP concentration, were assessed by commercial kits. Glucose consumption was measured using the glucose oxidase method and lactic acid concentration was detected by lactate dehydrogenase (LD) kit. Expression levels of PGC-1 and SIRT3 were much lower in breast cancer patients, compared with the normal controls. Overexpression of PGC-1α or SIRT3 both significantly promoted the apoptosis and inhibited the proliferation in MCF-7 and MDA-MB-231 cells. Additionally, PGC-1α or SIRT3 also induced the inhibition of glycolysis metabolism. Moreover, the expression of SIRT3 was positively regulated by PGC-1α. Silencing SIRT3 partly reversed the negative effects of PGC-1α on glycolysis metabolism.

These findings demonstrated that PGC-1α/SIRT3 regulated cell proliferation and apoptosis of breast cancer through altering glycolysis metabolism, which may provide novel therapeutic strategies for breast cancer.

Key words: PGC-1α; SIRT3; breast cancer; glycolysis; metabolism

Breast cancer is currently the most prevalent neoplastic disease that threatens to women’s health [1]. Statistical analysis shows that the incidence rate of breast cancer was the highest among new cancers in women, which account for more than 30% [2]. Despite much progress has been made in
the development of therapeutic strategies. The survival rate of breast cancer has not improved in recent decades [3]. Therefore, it is an urgent to identify novel biomarkers for the diagnosis, treatment and better understanding the potential mechanism of breast cancer tumorigenesis.

In normal cells, energy is mainly generated from the mitochondrial oxidative phosphorylation (OxPhos) pathway [4]. However, in tumor environment, the oxidative phosphorylation and mitochondrial respiratory function of tumor cells are irreversibly damaged. Whereas, glycolysis, the main energy acquisition method instead of OxPhos in most cancer cells even under aerobic concentrations, can be activated when mitochondrial respiratory function was impaired, which is known as the Warburg effect [5]. Such metabolic alterations from OxPhos to glycolysis could meet the increasing demand for energy of rapid proliferation cancer cells, as well as protecting cancer cells from oxidative stress since mitochondria were the main source of intracellular ROS, which is the is a fundamental property of cancer cells [6].

Peroxisome proliferator-activated receptor-gamma coactivators 1 α (PGC-1α) is the most extensively studied member from transcriptional coactivators, which could regulate the cell survival and proliferation of cancer cells by controlling mitochondrial biogenesis, and cellular metabolism [7]. Accumulating evidence has indicated the involvement of PGC-1α in the control of metabolic reprogramming in many kinds of cancer cells [8-10]. Recent studies have shown PGC-1α is both a tumor suppressor and promotor in cancer development [11]. In several cancers such as prostate cancer, PGC-1α could suppress cancer metastasis [12, 13]. However, deeper insights are still lack for role of PGC1-α in breast cancer.

Sirtuins, a family of NAD+-dependent histone deacetylases (HDACs), play essential roles in numerous physical processes including cell survival, apoptosis, metabolism, stress responses, cancer and aging [14, 15]. Among them, Siruin3 (SIRT3) is the most well characterized mitochondrial deacetylase, which targets many enzymes that involved in cellular metabolism, which is similar to PGC-1α. Besides, SIRT3 serves as an important regulator of energy homeostasis, and SIRT3 could coordinate the deacetylation of multiple mitochondrial proteins to regulate mitochondrial activity, which has significant implications for tumor growth [16-18]. Previous records have shown that PGC-1α could interact directly with SIRT1 [19], and PGC-1α overexpression could upregulate the
expression of SIRT3 in brown adipocytes [20]. However, the relationship between PGC-1α and SIRT3 and their roles in the proliferation of breast cancer is still unclear.

In the present study, we explored the potential roles of PGC-1α and SIRT3 in glycolysis metabolism of breast cancer cells. Our results revealed that overexpression of PGC-1α/SIRT3 decreased cell proliferation and induced apoptosis by inhibiting glycolysis in breast cancer cells.

Materials and Methods

Tissues. The 30 paired breast cancer and the adjacent normal tissue samples used in this study were obtained from tissue bank at North China University of Technology. The samples were frozen immediately after being surgically resected from the patients and stored in liquid nitrogen. All tissues were confirmed histologically and all patients received no chemotherapy or radiotherapy before the study. The study was approved by the Ethics Committee of North China University of Technology.

Cancer cell culture and transfection. MCF-7 and MDA-MB-231, human breast cancer cell lines, as well as normal cell line MCF10A, were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s minimal Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA). The media were supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 10,000 U penicillin/streptomycin. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

For cell transfection, adenoviral vectors expressing SIRT3-targeting shRNA (Ad-sh-SIRT3), Ad-sh-PGC-1α, and SIRT3 overexpression adenoviral vector (Ad-SIRT3), Ad-PGC-1α, as well as negative controls (NC) were all constructed and purchased from Fenghui Biotechnology (Changsha, China). After reaching 60-70% confluence, MCF-7 and MDA-MB-231 cells were transfected with the above vectors using Polyjet™ transfection reagent (SignaGen Laboratories, Gaithersburg, MD, USA), according to the manufacturer’s protocols. To inhibit glycolysis, glycolysis inhibitor 2-DG (10 nM) was used to treat the cells before transfection.

Cell viability assay. Cell viability was assessed using the CCK-8 assay. Briefly, Cells were seeded at density of 2.5 × 10⁵ in 96-well culture plates and were cultured overnight. After treatment of CPF for 24 h, cells were further cultured and cell viability was measured at 6 h, 12 h, 24 h and 48 h.
CCK-8 assay, 10 µl of CCK-8 solution was added to each well and cells were incubated at 37 °C for another 1 h. The absorbance of the solutions was detected at 450 nm by a SYNERGY-HT multiwell plate reader (Synergy HT, Winooski, VT, USA).

**Flow cytometry of cell apoptosis.** Apoptosis was determined by staining with Annexin V/PI (Beyotime, Haimen, China) as previously described. In brief, MCF-7 and MDA-MB-231 cells were incubated at a density of 2 × 10^5/well in 6-well plate with corresponding treatments, the cells were then washed with ice-cold PBS twice after removing the culture medium, and centrifuged at 800 rpm for 5 min, then stained with 5 µl of Annexin V and 5 µl of PI incubated with 100 µl of binding buffer for 15 min at RT in the dark. The fluorescent signal was further analyzed by flow cytometry after the reaction volume was raised to 500 µl by adding binding buffer.

**Hexokinase 2 and pyruvate kinase activity assay.** Hexokinase 2 and pyruvate kinase activities were determined using the Hexokinase 2 Activity Assay Kit and pyruvate kinase Activity Assay Kit (Abcam, Cambridge, UK) separately as previously described and followed by a fluorometric measurement (Ex/Em 540/590 nm).

**Glucose consumption assay.** Glucose consumption was measured using the glucose oxidase method as reported elsewhere [21]. Intracellular glucose was calculated by subtracting the glucose concentration in the medium from the total glucose concentration in blank wells.

**Lactic acid concentration.** For measurement of lactic acid concentration, cell supernatant was collected after centrifugation at 6000 × g for 10 min and the lactic acid concentration was determined by a lactate dehydrogenase (LD) kit (Nanjing Jiancheng biology Co., Ltd, Nanjing, China).

**NAD+/NADH analysis.** The ratio of NAD+/NADH was determined by a NAD/NADH fluorescence detection kit (Colorimetric, Abcam, USA) according to the manufacturer’s instruction.

**ATP concentration.** The mitochondrial ATP concentration was measured using ATP determination kit according to manufacturer’s instruction (Invitrogen, USA). The ATP concentration was normalized to total protein [22].

**RNA extraction and quantitative real-time PCR (qRT-PCR).** Briefly, total RNA was extracted using a TRIzol™ Plus RNA Purification Kit (Thermo Fisher Scientific, USA), and cDNA was generated by 500 ng RNA/sample using PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara,
Quantitative real-time PCR (qRT-PCR) was performed using an ABI 7000 Prism Step One plus detection system (Life Technologies, USA). The primer sequences used for qRT-PCR as follow: hsa-PGC-1α-F 5'-CACCAGCAAACACTGCTA-3'; hsa-PGC-1α-R 5'-GTGTGAGGAGGGTCATCGTT-3'; hsa-SIRT3-F 5'-CGTTGTGAAGGCCGACATTG-3'; hsa-SIRT3-R 5'-AGAAGGCTGGGCTCATTTG-3'; hsa-actin-F 5'-AGAAGGCTGGGCTCATTTG-3'; hsa-actin-R 5'-AGGGGCCATCCACAGTCTTC-3'. The gene expression levels were normalized using β-actin as an internal reference gene, and the average relative changes were calculated using the 2^-ΔΔCT method.

**Western blotting analysis.** After the treatments, cells were harvested and extracted into the cell lysis buffer supplemented with protease inhibitor (Beyotime, China) on ice. The cell lysates were centrifuged at 12,000 rpm for 20 min at 4°C after being sonicated. Then the supernatants were collected and protein concentration was determined using the BCA protein assay kit (Beyotime, China). Protein samples were diluted in 5× SDS-PAGE loading buffer and heated at 95°C for 5 min, then 25 μg protein was separated with 12% SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk powder dissolved in Tris-buffered saline containing and 0.1% Tween 20 (TBST) for 1 h at RT then incubated with primary antibodies at 4°C overnight. Primary antibodies were anti-PGC-1α (1:1000 dilution; #2178), and anti-SIRT3 (1:1000 dilution; #2627) (All purchased from Cell Signaling Technology). The membranes were then washed three times with TBST and incubated with secondary antibody for 1 h at RT. Images were captured and visualized according to the chemiluminescence system using the EU-88 image scanner. Signals were quantified and analyzed using the Image-Pro Plus 6.0 software (Media Cybernetics, Sarasota, USA).

**Statistical analysis.** Statistical analysis was performed using SPSS 22.0 software (IBM Corporation, NY, USA). The results were expressed as mean±standard deviation (SD). Comparisons were performed using one-way analysis of variance (ANOVA) and least-significant difference test. Differences were regarded as significant at p < 0.05.

**Results**
Overexpression of PGC-1α or SIRT3 inhibited proliferation of breast cancer cells. First, expression patterns of PGC-1α and SIRT3 were determined in the breast cancer tissues and cell lines. The results revealed that both mRNA and protein levels of PGC-1α and SIRT3 were downregulated in breast cancer tissues (Figure 1A). Besides, the expression of PGC-1α and SIRT3 was positively correlated in breast cancer tissues (Figure 1B). As shown in Figure 1C, expression of PGC-1α and SIRT3 in breast cancer cell lines MCF-7 and MDA-MB-231 cells was remarkably lower in normal cell line MCF10A cells. Then, to explore the roles of PGC-1α and SIRT3 in breast cancer, MCF-7 and MDA-MB-231 cells were both infected with Ad-PGC-1α or Ad-SIRT3, respectively. The data presented the high efficiency of transfection (Figures 2A, 2B). Cell viability was performed by CCK-8 assay and the data showed that PGC-1α or SIRT3 overexpression significantly inhibited proliferation in MCF-7 and MDA-MB-231 cells (Figure 2C). Moreover, PGC-1α or SIRT3 overexpression had no significant effect on the cell viability of MCF10A (Supplementary Figure S1A), which provide evidence that PGC-1α/SIRT3-mediated proliferation repression was only to cancer cells. Besides, PGC-1α or SIRT3 knockdown by their specific shRNA both notably promoted the cell viability of MCF-7 and MDA-MB-231 cells (Supplementary Figure S1B). In addition, cell apoptosis by flow cytometry also verified that overexpression of PGC-1α or SIRT3 markedly increased the apoptotic rate compared with the control group (Figure 2D). These findings implied that PGC-1α or SIRT3 may exert tumor suppressive function in the development of breast cancer.

Overexpression of PGC-1α or SIRT3 inhibited glycolysis metabolism in breast cancer cells. Due to the key roles of PGC-1α and SIRT3 in the regulation of energy metabolism, we then wondered how glycolysis metabolism pattern was influenced by PGC-1α and SIRT3 in breast cancer cells. We determined the activities of hexokinase2 and pyruvate kinase, which are two important enzymes involved in glycolysis. The results suggested that both overexpression of PGC-1α and SIRT3 could significantly decrease the activities of hexokinase2 and pyruvate kinase in MCF-7 and MDA-MB-231 cells compared with the control group (Figure 3A). Meanwhile, glucose consumption and lactic acid concentration were also examined. We also found that overexpression of PGC-1α or SIRT3 remarkably reduced the glucose consumption and lactic acid production (Figures 3B, 3C). Besides, overexpression of PGC-1α or SIRT3 increased the ratio of
NAD+/NADH and decreased the ATP concentration in breast cancer cells (Figures 3D, 3E). All these results illuminated both PGC-1α or SIRT3 might decrease the proliferation of breast cancer cells via inhibiting glycolysis metabolism in breast cancer cells.

**PGC-1α positively regulated the expression of SIRT3.** Then, we further investigated the underlying mechanism involving the inhibitory effects of PGC-1α on breast cancer cells. The mRNA and protein levels of PGC-1α and SIRT3 were determined in cells transfected with Ad-PGC-1α or sh-PGC-1α. The results showed that upregulation of PGC-1α significantly increased both mRNA and proteins levels of SIRT3, compared with control group (Figures 4A, 4B). However, silencing PGC-1α led to opposite results, with remarkably decreased level of SIRT3 (Figures 4C, 4D), suggesting that PGC-1α could positively regulate the expression of SIRT3. However, SIRT3 overexpression did not exert ant effect on PGC-1α expression (Supplementary Figure S1C). These data suggested that SIRT3 may act as a downstream effector of PGC-1α.

**SIRT3 involved in PGC-1α-induced proliferation of breast cancer cells.** To further illuminate the underlying mechanisms for the interaction between SIRT3 and PGC-1α in breast cancer, we infected MCF-7 and MDA-MD-231 cells by adenoviral vectors to upregulate PGC-1α and meanwhile silencing SIRT3 expression. As shown in Figures 5A and 5B, the expression of SIRT3 was remarkably enhanced by overexpression of PGC-1α. However, silencing SIRT3 has little effects the PGC-1α level, indicating that SIRT3 was a downstream protein of PGC-1α. Further, knockdown of SIRT3 partially reversed the inhibition of Hexokinase2 and pyruvate kinase activity by PGC-1α overexpression (Figure 5C). Meanwhile, the glucose consumption and lactic acid production were also remarkably elevated by SIRT3 downregulation, compared with Ad-PGC-1α group (Figures 5D, 5E). Additionally, silencing SIRT3 also significantly decreased NAD+/NADH ratio while increased ATP concentration compared with Ad-PGC-1α group (Figures 5F, 5G).

Next, we detected the effect of inhibition of SIRT3 in PGC-1α overexpression cells on both cell viability and apoptosis. As expected, cell viability was significantly increased in co-transfection with Ad-sh-SIRT3 and Ad-PGC-1α cells compared to cells transfected with Ad-PGC-1α alone, and this effect was reversed by the glycolysis inhibitor 2-DG (Figure 5H). Moreover, PGC-1α knockdown could also rescue the repression of proliferation induced by SIRT3 overexpression in MCF-7 and MDA-MD-231 cells (Supplementary Figure S1D). On the contrary, inhibition of SIRT3...
remarkably decreased the cell apoptosis rate in co-transfection with Ad-sh-SIRT3 and Ad-PGC-1α cells compared with cells transfected with Ad-PGC-1α alone, and this effect was also reversed by treatment of 2-DG (Figure 4I). Taken together, these results illuminated that PGC-1α suppressed glycolytic metabolism by activating SIRT3, thereby inhibiting breast cancer cells proliferation and inducing apoptosis.

Discussion

Breast cancer is now the leading cause of death among female patients among the world [23]. In order to support the rapid growth and proliferation, numerous cancer cells including breast cancer cells utilize aerobic glycolysis rather than oxidative phosphorylation as the main energy source, namely Warburg effect [24, 25]. Altered energy metabolism plays an essential role in the development of cancer. In this paper, we determined the relationship between PGC-1α and SIRT3, and how they contributed to the development of breast cancer cells. Our results revealed that PGC-1α could suppress glycolytic metabolism by activating SIRT3 to moderate the proliferation of breast cancer cells.

PGC-1α, a member of transcriptional co-activators family [26], plays a key role in the regulation of cellular metabolism and cancer development [27-30]. The relationship between PGC-1α and breast cancer aggressiveness remains controversial. Some studies have demonstrated that the expression of PGC-1α is enriched in breast cancer cells [31, 32]. However, PGC-1α also promoted the sensitivity of anti-folate therapy in breast cancer [33]. In most cancers, PGC-1α is considered as a tumor suppressor rather than promotor. It has been reported that PGC-1α could suppress the cell metastasis in prostate cancer, and the expression of PGC-1α led to impaired tumor growth and enhanced sensitivity to cytotoxic therapies in clear cell renal cell carcinoma [12, 34]. In this study, we found that overexpression of PGC-1α led to significant decrease of cell viability and increase of apoptotic rate of breast cancer cells via inhibiting glycolysis. Furthermore, the inhibition of SIRT3 led to opposite result, which was reversed by glycolysis inhibitor 2-DG.

As is widely accepted that most cancer cells change the traditional cellular metabolism to use mainly glycolysis rather than oxidative phosphorylation to gain cellular energy [35]. In glycolysis, hexokinase2 catalyzes phosphorylation of glucose to glucose-6-phosphate at the initial step.
Selective inhibition of hexokinase2 activity could reduce the production of glucose-6-phosphate, thereby blocking the glycolysis and inhibiting the proliferation of cancer cells [36, 37]. Pyruvate kinase, catalyzes transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) at the final step of glycolysis [38]. Lack of pyruvate kinase could slow down the process of glycolysis [39]. And overexpression of pyruvate kinase was observed in many tumors, leading to the shift of energy metabolism towards glycolysis [40]. In the present study, we observed that overexpression of PGC-1α significantly decreased hexokinase2 and pyruvate kinase activities, as well as decrease the levels of glucose consumption, ATP concentration and lactic acid production, and increased the level of NAD+/NADH ratio in breast cancer cells. In contrast, hexokinase2 and pyruvate kinase activities were increased by inhibition of SIRT3 in breast cancer cells, demonstrating that PGC-1α exerts potential role in enhancing glycolysis in breast cancer cells through regulation of SIRT3.

SIRT3, a major mitochondrial deacetylase, plays important role in cellular metabolism [15]. Similar to PGC-1α, SIRT3 has been suggested to be both a tumor promoter and suppressor [41]. It was found that elevated level of SIRT3 was associated with many kinds of cancer cells, including oral cell carcinoma and node-positive breast cancer [42, 43]. However, studies also found that SIRT3 could suppress cancer development in chronic myelogenous and hepatoma carcinoma [44]. Our data suggested that knockdown of PGC-1α could inhibit the expression of SIRT3, and PGC-1α overexpression could stimulate the expression of SIRT3, suggesting the positive regulation of PGC-1α on SIRT3. Several studies also showed the relationship between PGC-1α and SIRT3. It has been demonstrated that PGC-1α could regulate the expression of SIRT3 in diabetes, ischemia-induced oxidative stress and mitochondrial biogenesis [45-47]. In our findings, we further presented that knockdown of SIRT3 could partly reversed PGC-1α-mediated glycolysis in breast cancer cells.

In conclusion, our results revealed that PGC-1α could activate SIRT3 to suppress cell proliferation by inhibited glycolysis metabolism, which may serve as a potential therapeutic target in breast cancer in the future. Meanwhile, the current study also has some limitations. In tissue samples, we didn’t distinguish the subtypes of breast cancer. In in vitro studies, more underlying molecular
mechanisms for PGC-1α/SIRT3 axis in breast cancer are still unclear and need further researches to reveal.

References


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**Figure Legends**

Figure 1. Effects of PGC-1α or SIRT3 overexpression on breast cancer cells. A) mRNA and protein expression levels of PGC-1α or SIRT3 were determined in breast cancer tissues by RT-qPCR and western blotting, respectively. B) The correlation between PGC-1α and SIRT3 in breast cancer tissues. C) Expression of PGC-1α and SIRT3 in normal cell line MCF10A cells and breast cancer cell lines was determined by western blotting and qRT-PCR. Data are presented as mean±SD. Statistical significance compared with the control is indicated by *p < 0.05, **p < 0.01 and ***p < 0.001.
Figure 2. Effects of PGC-1α or SIRT3 overexpression on breast cancer cells. A) mRNA expression of PGC-1α or SIRT3 by qRT-PCR in cells transfected with ad-SIRT3, or ad-NC. B) Protein expression of PGC-1α or SIRT3 by western blotting in cells transfected with ad-SIRT3, or ad-NC. C) Cell viability was determined by the CCK-8 assay. D) Cell apoptosis rate was determined with Annexin V-FITC/PI staining by flow cytometry. Data are presented as mean±SD. Statistical significance compared with the control is indicated by *p < 0.05, **p < 0.01 and ***p < 0.001.

Figure 3. Effects of PGC-1α or SIRT3 on glycolysis metabolism in breast cancer cells. A) Hexokinase 2 and pyruvate kinase activities were determined as described in the Materials and Methods. B) Glucose consumption was measured using the glucose oxidase method. C) Lactic acid concentration was determined by LD kit. D) NAD+/NADH ratio and E) ATP concentration were measured using commercial kits. β-actin Data are presented as mean±SD. Statistical significance compared with the control is indicated by *p < 0.05 and **p < 0.01.

Figure 4. Overexpression of PGC-1α increased the expression of SIRT3. A) mRNA and B) protein levels of PGC-1α, SIRT3 and β-actin (loading control) were determined in cells transfected with Ad-PGC-1α by qRT-PCR and western blotting, respectively. C) mRNA expressions and D) protein levels of PGC-1α, SIRT3 and β-actin (loading control) were determined in cells transfected with Ad-sh-PGC-1α by qRT-PCR and western blotting, respectively. Data are presented as mean±SD. Statistical significance compared with the control is indicated by *p < 0.05 and **p < 0.01.

Figure 5. SIRT3 involved in PGC-1α-induced proliferation of breast cancer cells. A) mRNA and B) protein expression levels of PGC-1α, SIRT3 and β-actin (loading control) were determined by RT-qPCR or western blotting. C) Hexokinase 2 and pyruvate kinase activities were determined as described in the Materials and Methods. D) Glucose consumption was measured using the glucose oxidase method. E) Lactic acid concentration was determined by LD kit. F) NAD+/NADH ratio and G) ATP concentration were measured using commercial kits. H) Cell viability was determined by the CCK-8 assay. I) Cell apoptosis rate was determined with Annexin V-FITC/PI staining by flow
cytometry. Data are presented as mean±SD. Statistical significance compared with control, *p < 0.05, **p < 0.01.

Supplementary Figure Legends

Supplementary Figure S1. A) CCK-8 assay was performed to assess the cell viability of PGC-1α or SIRT3 overexpression on the normal cell line MCF10A cells. B) The effects of PGC-1α or SIRT3 knockdown on cell viability were examined using CCK-8 assay. C) Cell viability was determined by the CCK-8 assay. D) qRT-PCR assay was subjected to evaluate the expression of PGC-1α within SIRT3 overexpression. Data are presented as mean±SD. Statistical significance compared with control, *p < 0.05, **p < 0.01.
Fig. 2

(A) Relative PGC-1α levels.

(B) Western blot analysis for PGC-1α and β-actin. SIRT3 and p-actin.

(C) Cell viability assay over time for MCF7 and MDA-MB-231.

(D) Flow cytometry analysis for apoptosis rate.