PPARγ Agonist Pioglitazone Inhibits PDGF-induced Pulmonary Artery Smooth Muscle Cells Proliferation and Migration via Modulating TERT

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Research

Keywords: Pulmonary artery hypertension, Telomerase, Telomerase reverse transcriptase

Posted Date: February 24th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-233120/v1

Abstract

Background: Vascular remodeling is a significant feature of pulmonary artery hypertension (PAH), characterized by abnormal proliferation and migration of pulmonary arterial smooth muscle cells (PASMCs). Telomerase reverse transcriptase (TERT) as a determinant factor for controlling telomerase activity has been proved to be associated with cell proliferation. This study aims to explore whether TERT participates in the proliferation of PASMCs and the underlying molecular mechanism.

Methods: Primary PASMCs of SD rat were used in this experiment. the proliferation and migration of cells were detected by Cell Counting Kit-8 and transwell assay, respectively. The telomerase activity was determined by Rat TE ELISA KIT. The siRNA transfection was conducted to silence the expression of c-MYC. the protein levels of p-Akt, c-MYC and TERT were determined through western blotting.

Results: We found that PDGF upregulated TERT expression and telomerase activation by activation of Akt and upregulation of c-MYC in PASMCs. Inhibition of Akt by LY294002, knockdown of c-MYC by siRNA or suppression of telomerase activity by BIBR1532 repressed PDGF-induced PASMCs proliferation and migration. Furthermore, activation of Peroxisome proliferator-activated receptor γ (PPARγ) by pioglitazone suppressed PDGF-induced TERT expression and telomerase activation, leading to inhibition of PASMCs proliferation and migration.

Conclusion: our work demonstrates that TERT mediates PDGF-induced proliferation and migration of PASMCs. In addition, activation of PPARγ inhibits these processes via Akt/c-MYC/TERT pathway.

Background

Pulmonary arterial hypertension (PAH) is a chronic and multidisciplinary disorder characterized by a resting mean pulmonary arterial pressure ≥25 mmHg, pulmonary artery wedge pressure ≤15 mmHg, and PVR >3 Wood units. The main pathobiology of pulmonary hypertension includes pulmonary vasoconstriction, concentric vascular remodeling and thrombosis in situ[1]. Mounting evidence indicates that excessive proliferation and migration of pulmonary arterial smooth muscle cells lead to development of vascular remodeling in PAH, but the underlying mechanism is still elusive. Thus, exploring novel mechanisms is critical for reversing the progress of PAH and providing specific targets for treating PAH.

Telomerase is characterized as a ribonucleic acid-protein complex TERT, which consists of the non-coding Telomerase RNA Component (TERC), telomerase-associated protein 1 (TEP1) and telomerase reverse transcriptase (TERT)[2,3]. Studies indicate TERT is a rate-limiting factor for controlling telomerase activity. In addition, disfunction of TERT and activation of telomerase are related in various diseases including cardiovascular disease[4], type 2 diabetes mellitus[5], cancer [6], chronic obstructive pulmonary disease[7] as well as PAH [8,9].However, the underlying molecular mechanism that TERT participated in the development of PAH remains unclear.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of nuclear hormone receptor superfamily that involves in metabolic homeostasis, cellular proliferation and vascular protection[10]. Activated PPARγ protects tissues against excessive lipid overload and controls insulin sensitivity. Thus, pioglitazone as a synthetic PPAR-agonist is widely used to treat type 2 diabetes[11]. Some research has found that activated PPARγ is a protective modulator and participates in the regulation of PAH development[12]. Our previous study also found that activation of PPARγ in rat results in the amelioration of PAH[13]. Therefore, we consider whether PPARγ is involved in the expression of TERT and telomerase activity in PAH. To investigate this hypothesis, we conduct the study to determine the novel molecular mechanism of PPARγ in PAH and explore cross-link between PPARγ and TERT.

Materials And Methods

Cell culture

Primary PASMCs were prepared from main pulmonary arteries of male Sprague-Dawley rats (100–150 g) according the previously reported method[14]. All animals care and procedures were conformed to Xi'an Jiaotong University Animal Care Policy and complied with the Guide for the Care and Use of Laboratory Animals[15] . All experimental protocols used throughout current study were approved by the laboratory Animal Care and Use Committee of Xi'an jiaotong University. Sprague-Dawley rats were anesthetized and lungs were removed rapidly. The main pulmonary arterials were isolated without connective tissues and endothelium, next, we wiped off adventitia and intima repeatedly. The residual pulmonary arteries were minced into small pieces (0.5–1mm3) and transferred to a culture flask. Cells were incubated with Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Isle, NY, USA) containing 10% fetal bovine serum (FBS; Sijiqing, HangZhou, China), 100 U/ml penicillin and 100μg/ml streptomycin in a humidified atmosphere of 5% CO2 and 95% air at 37℃. At 80% confluence, cells were trypsinized using 0.25% trypsin (Invitrogen, Carlsbad, CA, USA) and the 3th to 6th generation cells were used through the experiment. For minimizing the effect of serum, cells were cultured in 1% FBS-DMEM overnight before different treatment. PDGF (10 ng/ml) (Gibco, Carlsbad, USA) was conducted to stimulate cells. Pioglitazone (10 μmol/l) (Cayman Chemical Company, Michigan, USA), was used to active PPARγ. LY294002 (25μmol/l) (MCE, Monmouth, USA) was used to inhibit the phosphorylation of Akt and BIBR1532 (5 μmol/l) (APExBIO Techonology, Houston, TX, USA) was conducted to impede the activity of telomerase.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8) was performed to detect cell proliferation qualitatively according to the manufacturer's protocol. Primarily, PASMCs were plated into 96-well microplates at a concentration of 5×103 cells/well. After different treatment, each well was added to 10 μL of CCK-8 solution for 2h. Finally, we measured the absorbance at 450 nm using microplate reader.

Small interfering RNA transfection

c-MYC expression was knocked down by the c-MYC siRNA purchased from GenePharma (Shanghai, China). PASMCs were incubated in 6-well plates until reaching approximately 30-40% confluence. Based on the manufacturer's instructions, we diluted siRNA and Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) with serum-free DMEM at room temperature for 5 min. cells were treated with the mixture of Lipofectamine™ 2000 and siRNA after incubation for 20 min. Finally, PASMCs were determined for proliferation and harvested for Western blotting.

Transwell assay

Cell migration was measured by transwell chamber with 8.0-μm pore membrane (Corning, Lowell, MA, USA). After 48h transfection, 200 μl of cell suspension (2.5×10 5 cells/ml) was added into the upper chamber in the serum-free medium. 10% FBS-DMEM containing PDGF was added to the lower compartment with or without indicated preceding inhibitors. After incubation at 37°C for 12 h, the inserts were taken out and fixed with 4% (w/v) paraformaldehyde for 20 min. Non-migrated cells in the upper chamber were wiped off using a cotton swab. Then, migrated cells in the lower surface of the membrane were stained with 0.1% crystal violet and quantified by counting cells under a microscope.

Telomerase activity assay

The telomerase activity was determined by Rat TE ELISA KIT(FANKEW, Shanghai, China) according to the manufacturer's introduction. Cells lysates acquired by ultrasonication was added into 96-wells plate and covered with a strip and incubated for 30 mins at 37 °C. after Washing, HRP- antibody working solution was added into per well and incubated for 30 mins. Then, the TMB Substrate reacted with HRP- antibody working solution. Finally, the optical density was measured by a microplate reader set to 450 nm.

Immunoblotting

Cells lysates were obtained by RIPA lysis buffer (HEART, Xi'an, Shaanxi, China). Extracted proteins counting loading buffer were subjected to 10% SDS-PAGE gel and transferred onto PVDF membranes (Millipore, Billerica, MA). Membranes were blocked in PBS with 3% bovine serum albumin and incubated with primary antibodies at 4 °C overnight. The following antibodies were used according to the manufacturer's protocols: Monoclonal antibodies against Akt (#4691), phospho-Akt (Ser473) (#13008) and c-MYC (#9402) (Cell Signaling Technology, 1:1000 dilution), and β-actin (#YM3028, ImmunoWay, 1:2000 dilution), and polyclonal antibody against telomerase reverse transcriptase (#ab191523, Sigma, 1:1000 dilution). After washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, the band was visualized by ChemiDoc XRS system and analyzed by Image-J software.

Statistical analysis

Data were represented as mean ± standard error of the mean (SEM) and analyzed by GraphPad Prism 7.0 software. differences between two groups were performed with a Student's *t*-test. differences among

multiple groups were determined using the one-way ANOVA followed by the Tukey test for multiple comparisons. P<0.05 was considered statistically significant.

Results

PDGF induces PASMCs proliferation

To explore the effect of PDGF on PASMCs proliferation, cell viability was measured using CCK-8 at different concentrations (0,3,10,30,100 ng/ml) or different times (0, 12, 24,48,72 h). Figure 1a shows that cell viability was enhanced with increasing concentration of PDGF and 10 ng/ml PDGF triggers a 1.29-fold increase at 24 h compared control ($P < 0.05$). Figure 1b indicates that PDGF promoted cell proliferation time-dependently and cell viability reached 1.71-fold increase at 72 h over control ($P < 0.05$). The observed results reveal that PDGF induces PASMCs proliferation in a dose and time-dependent manner.

PDGF activates PI3K/Akt signaling pathway and upregulates c-MYC and TERT expressions

PI3K/Akt signaling pathway as a positive regulator mediates PDGF-induced PASMCs proliferation [16]. To understand the mechanisms underlying PDGF-induced PASMCs proliferation, we examined the phosphorylation level of Akt, protein levels of c-MYC and TERT using western blotting. As shown in Figure 2a, b and c, 10 ng/ml PDGF significantly increased the phosphorylation level of Akt, c-MYC and TERT expressions to 1.50-fold, 1.63-fold and 1.84-fold over control, respectively ($P < 0.05$). These results suggest that PDGF activates PI3K/Akt signaling pathway and increases protein levels of c-MYC and TERT in PASMCs.

PI3K/Akt signaling pathway mediates PDGF-induced c-MYC and TERT expressions

It has reported that the PI3K/Akt signaling pathway promotes the transcription of TERT and cell proliferation by suppressing the function of MAX dimerization protein1in MCF-7 Cell Line [17]. Here, to investigate whether it exists in PASMCs, cells were pre-treated with PI3K/Akt inhibitor LY294002 for 30 min and then stimulated with PDGF. We found that LY294002 significantly inhibited Akt phosphorylation to 1.11-fold over control ($P < 0.05$, versus PDGF-treated group Figure 3a). Moreover, the protein level of c-MYC was declined to 0.89-fold over control and TERT expression was dropped to 1.03-fold compared with control ($P < 0.05$, versus PDGF-treated group, Figure 3b and c). These results indicate that activation of Akt is responsible for PDGF-induced c-MYC and TERT expressions in PASMCs.

TERT expression is regulated by c-MYC

c-MYC promotes TERT expression through binding to the TERT proximal promoter in neoplastic cells[18]. To clarify whether c-MYC regulates TERT expression in PASMCs, c-MYC was knocked down by siRNA and protein level of TERT was measured by western blotting. As shown in Figure 4a, the protein level of c-MYC was reduced to 29% after transfection of c-MYC siRNA in PASMCs for 48 h, meanwhile, control siRNA had no effect on the c-MYC protein level. Figure 4b indicates that PDGF stimulation increased TERT protein

level to 1.96-fold over control ($P < 0.05$), while transfection of c-MYC notably reduced PDGF-induced TERT protein level to 1.14-fold compared with control ($P < 0.05$, versus PDGF-treated group). These results suggest the crucial role of c-MYC in PDGF-induced TERT expression.

Activation of PPARγ suppresses the effects of PDGF on Akt/c-MYC/TERT axis

To understand the effect of PPARγ on the process of PDGF-induced cell proliferation, cells were pretreated with pioglitazone (10 mM) as PPARγ agonist for 30min and then stimulated with PDGF for 24 h. As shown in Figure 5a, pioglitazone suppressed the activation of Akt induced by PDGF and the protein level of p-Akt was declined to 1.07-fold compared with control ($P < 0.05$, versus PDGF-treated group). Figure 5b and C indicates that cells pretreated with pioglitazone dramatically reduced the expression of c-MYC from 1.55-fold to 0.94-fold over control and TERT expression from 1.61-fold to 0.97-fold over control ($P < 0.05$, versus PDGF-treated group). All results suggested Activation of PPARγ inhibits PDGF-induced expression of p-Akt, c-MYC and TERT.

PDGF induces telomerase activation via Akt/c-MYC/TERT pathway

To identify whether telomerase activity in PASMCs was triggered by PDGF and was directly mediated via Akt/c-MYC/TERT pathway, cells were incubated with PDGF for 24 h. Figure 6 shows that PDGF significantly increased telomerase activation to 1.45-fold compared with control ($P < 0.05$). it also indicated that treatment cells with LY294002, BIBR1532, transfection of c-MYC-siRNA or pioglitazone reduced telomerase activation to 1.02-fold, 1.02-fold, 0.90-fold and 0.98-fold over control respectively ($P < 0.05$, versus PDGF-treated group). These results demonstrate PDGF activates telomerase through Akt/c-MYC/TERT signaling and activation of PPARγ impedes this process.

PDGF-induced telomerase activation promotes cell proliferation and migration by Akt/c-MYC/TERT pathway

According to the above data, we hypotheses that telomerase activation might mediate cell proliferation and migration by Akt/c-MYC/TERT pathway. We then examined cell viability by CCK-8 assay. As shown in Figure 7a, PDGF increased cell viability to 1.55-fold compared with control ($P < 0.05$), conversely BIBR1532 reversed this affect and attenuated the cell viability to 1.14-fold compared with control ($P < 0.05$, versus PDGF-treated group) due to the inhibition of telomerase activation. Meanwhile, pre-treatment cells with LY294002, pioglitazone or transfection of c-MYC-siRNA declined cell viability to 1.17-fold, 1.12-fold, 1.18-fold over control, respectively ($P < 0.05$, versus PDGF-treated group). Consistently, Figure 7b suggested that PDGF stimulation promoted cells migration, whereas BIBR1532 reversed PDGF-induced PASMCs migration and similar effects were observed in LY294002, c-MYC-siRNA and pioglitazone groups. All results prove the hypothesis that telomerase activation facilitates PASMCs proliferation and migration via Akt/c-MYC/TERT pathway

Discussion

In this study, we identified that PDGF promoted PASMCs proliferation and migration via Akt/c-MYC/TERT pathway. TERT expression and telomerase activity participated in the proliferation and migration in PASMCs, suggesting that TERT might be involved in the pathogenesis of PAH. Our study also found that activation of PPARγ suppressed TERT expression and telomerase activity, and then inhibited proliferation and migration of PASMCs, which might be the therapeutic target for treatment of PAH.

PDGF family is considered as growth factors composed of five different disulphide-linked dimers[19]. Among these isoforms, PDGF-BB involves in intracellular activation via binding to two receptor tyrosine kinases, PDGF receptors α and β[20]. Accumulating evidence has reported that PDGF-BB is a crucial stimulant for VSMC proliferation and migration in vitro [21,22]. in addition, continuous PDGFR activation accelerates the development of pulmonary vascular remodeling and pulmonary arterial hypertension in PAH mice [23,24]. In this work, our study demonstrated that PDGF-BB promoted PASMCs proliferation and migration.

c-MYC, an oncogenic transcription factor, exerts diverse biological activities such as cell proliferation, apoptosis and cellular metabolism. Previous study has indicated that c-MYC promotes myoblast proliferation and muscle fibre hypertrophy[25]. In our study, we found that c-MYC mediated PDGF-induced proliferation and migration of PASMCs. In cellular processes, c-MYC acts as the integrator at both transcriptional and post-transcriptional levels[26]. It has reported that c-MYC activates transcription of various target genes by binding to E-box sequences located on the promoter regions [25]. In HaCaT cells, chromatin immunoprecipitation assay demonstrates that c-MYC modulates transcriptional activity of TERT through enhancing on the TERT promoter[27]. In the present study, we found that knockdown of c-MYC declined PDGF-induced TERT expression and telomerase activation, indicating that c-MYC mediated the upregulation of PDGF-induced TERT expression and telomerase activation in PASMCs.

Telomerase is consisted of noncoding TTAGGG nucleotide repeats, preventing the ends of chromosomes from deterioration[28].Telomerase reverse transcriptase (TERT), a catalytic component of telomerase, remains telomere homeostasis by lengthening telomeric DNA[29]. Study shows that BIBR1532 is a selective telomerase inhibitor[30], which corresponded with our result that BIBR1532 inhibited telomerase activity in PASMCs. In most human somatic cells, TERT gene is suppressed and telomerase activity is inhibited by regulation of the TERT promoter[31]. However, upregulation of TERT and telomerase activation occur during progress in PAH[8]. Consistently, our results showed that PDGF increased the expression of TERT in PASMCs and Inhibition of TERT through BIBR1532 repressed PDGF-induced PASMCs proliferation and migration, suggesting TERT might involve in the process of PASMCs abnormal proliferation in PAH.

PPARγ as a ligand-activated transcription factors controls the expression of genes which are essential in cell differentiation and diverse metabolic processes[32]. In adipocytes, hepatocytes and skeletal muscle cell, PPARγ indirectly increases insulin-stimulated glucose uptake. Thus, PPARγ agonists is recommended as anti-diabetic drugs in the treatment of type 2 diabetes[33]. PPARγ is highly expressed not only in adipose tissue, but also in vascular smooth muscle cells. Our previous studies have suggested that

PPARγ activation inhibits proliferation in PASMCs, thereby reverses PAH[34]. In this study, we identified pioglitazone as a PPARγ agonist suppressed activation of Akt, c-MYC expression, TERT expression and telomerase activation in PASMCs. At the same time, activated PPARγ inhibited proliferation and migration of PASMCs induced by PDGF.

Conclusions

Taken together, we have demonstrated that PDGF promotes PASMCs proliferation and migration via Akt/c-MYC/TERT pathway. In addition, activation of PPARγ suppresses PASMCs proliferation and migration by targeting on this pathway. In light of these findings, TERT might become a potential therapeutic target to inhibit and reverse the development of vascular remodeling in PAH.

Abbreviations

PAH: Pulmonary artery hypertension

PASMCs: Pulmonary arterial smooth muscle cells

PDGF: Platelet-derived growth factor

TERC: Telomerase RNA Component

TEP1: telomerase-associated protein 1

TERT: telomerase reverse transcriptase

PPARγ: Peroxisome proliferator-activated receptor γ

DMEM: Dulbecco's Modified Eagle Medium

FBS: fetal bovine serum

CCK-8: Cell Counting Kit-8

SEM: mean ± standard error of the mean

Declarations

Acknowledgements

The authors thank the National Natural Science Foundation for their support.

Funding

This work was supported by the National Natural Science Foundation of China (Grant numbers 81800052) and the Natural Science Foundation of Shaanxi Province (2019JQ390)

Contributions

Xinming Xie were responsible for study concept and design. Qianqian Zhang contributed to conduct experiments. Wei Feng, Wenhua Shi and Jian Wang was responsible for data analysis. All authors contributed to the drafting of this manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have stated that there are no conflicts of interest.

Availability of data and material

Not applicable.

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Figures

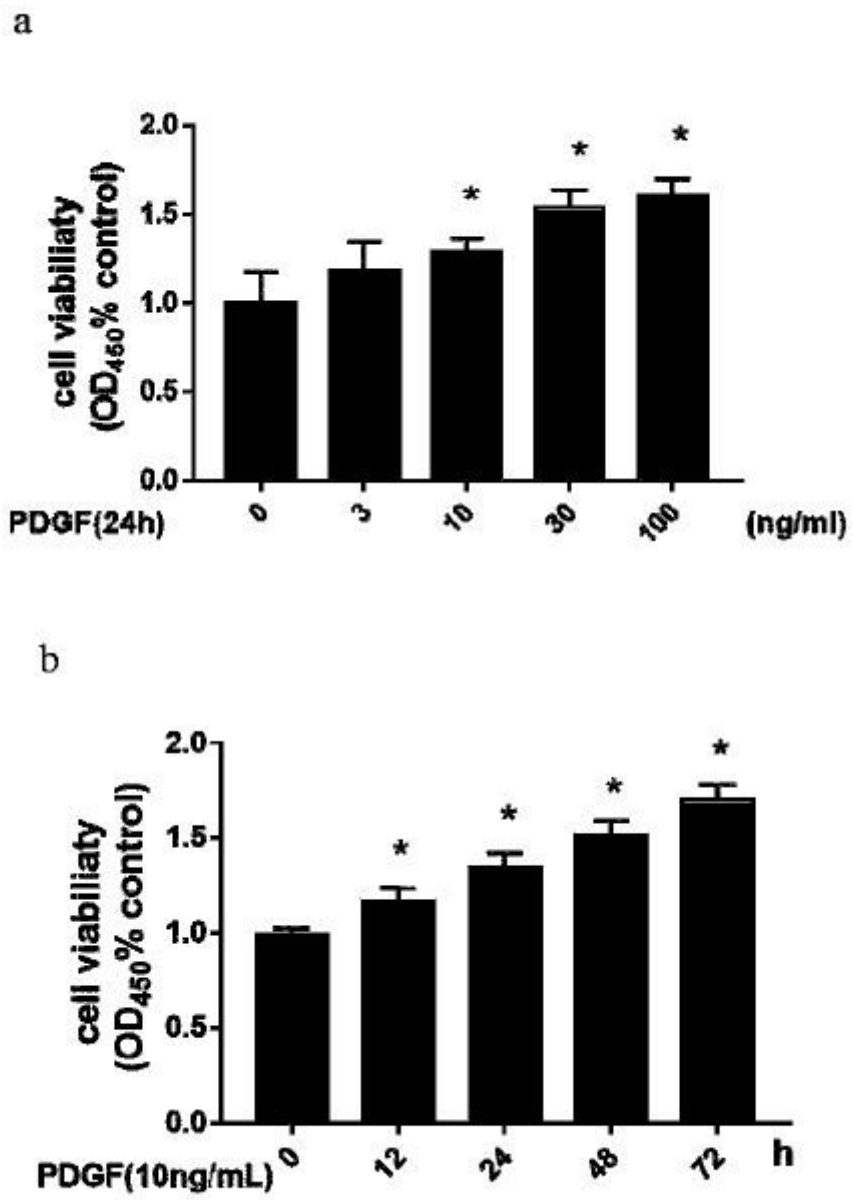

Figure 1

PDGF promotes PASMCs proliferation. (a) Cells were stimulated with PDGF for 24 h, which concentration ranged from 0 to 100 ng/ml. Cell proliferation was detected by CCK-8 assay (n=6 per group). (b) 10 ng/ml PDGF stimulated PASMCs for different time (0, 12, 24, 48, 72h). Cell proliferation was detected by CCK-8 assay (n=6 per group). * $P < 0.05$ versus control.

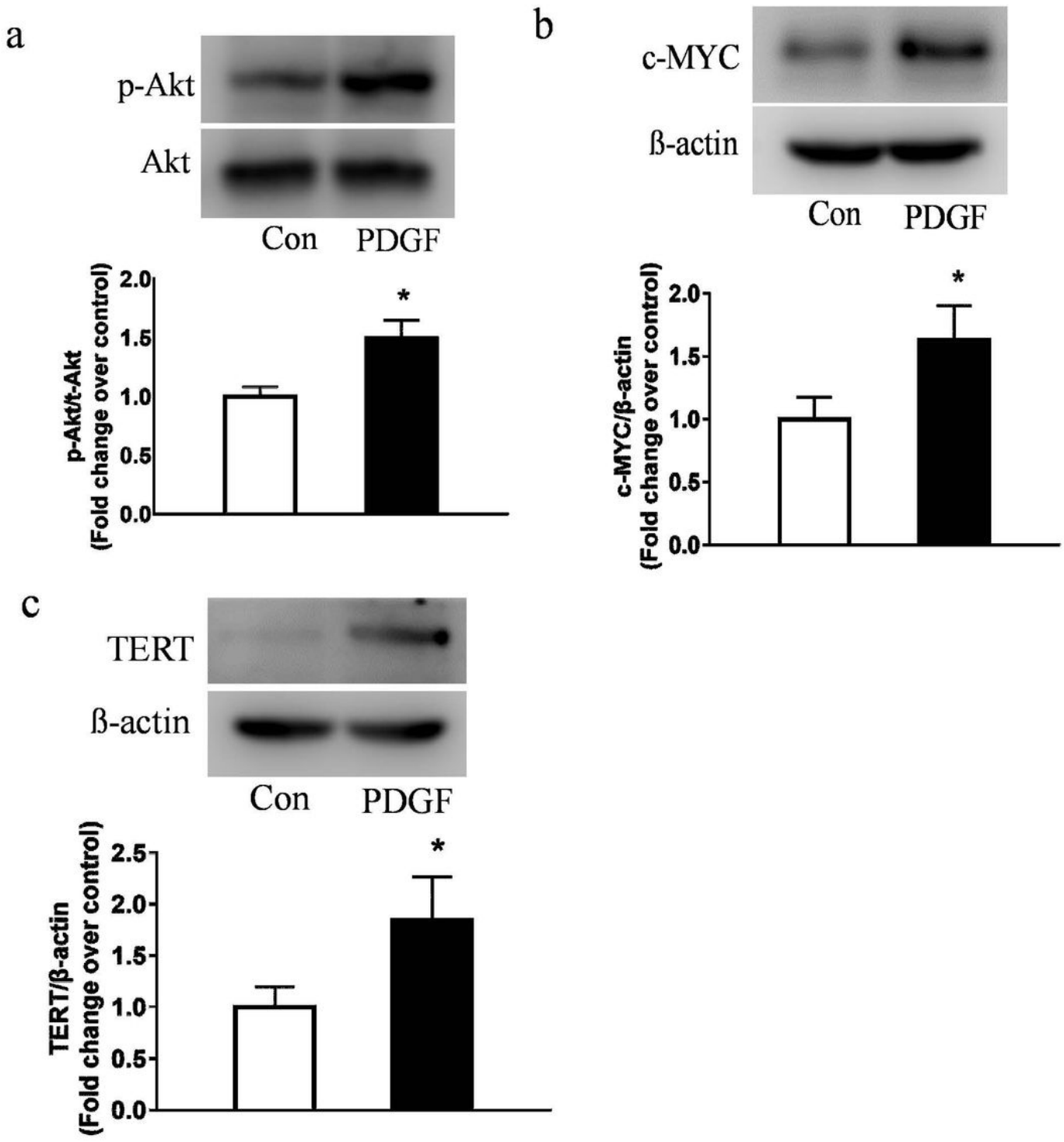

Figure 2

PDGF induces the activation of Akt and upregulation of c-MYC and TERT. 10 ng/ml PDGF treated cells for 24 h. The expression of p-Akt (a), c-MYC(b) and TERT(c) were determined by western blotting. t-Akt or β-actin served as a loading control (n=3 per group, * P < 005 versus control).

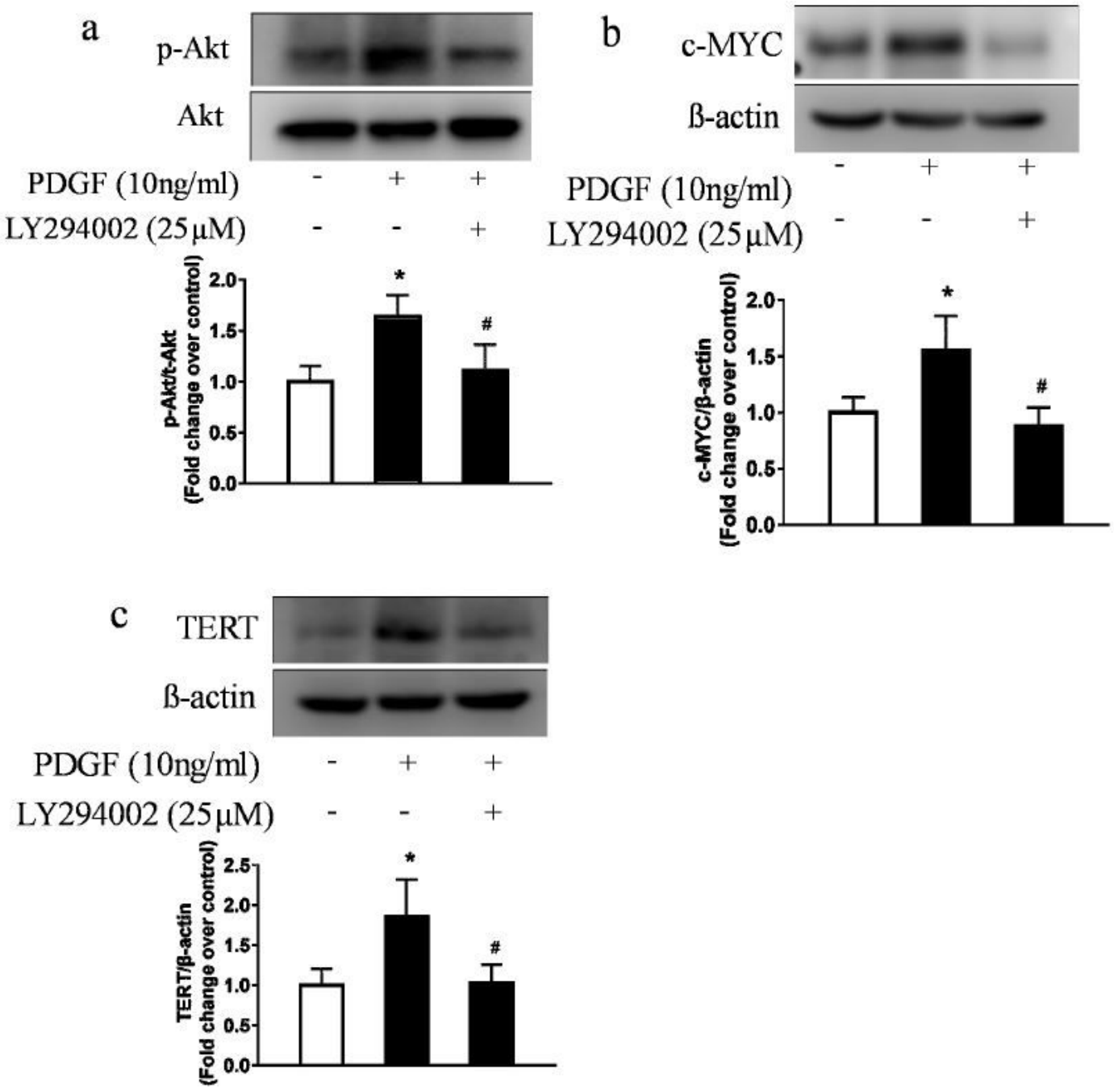

Figure 3

Akt mediates the PDGF-induced upregulation of c-MYC and TERT. LY294002 was pre-treated cells for 30 min before PDGF stimulation. The expression of p-Akt(a), c-MYC(b) and TERT(c) were measured using western blotting. t-Akt or β-actin served as loading control. (n=3 per group). * $P < 0.05$ versus control. # $P < 0.05$ versus PDGF-treated group.

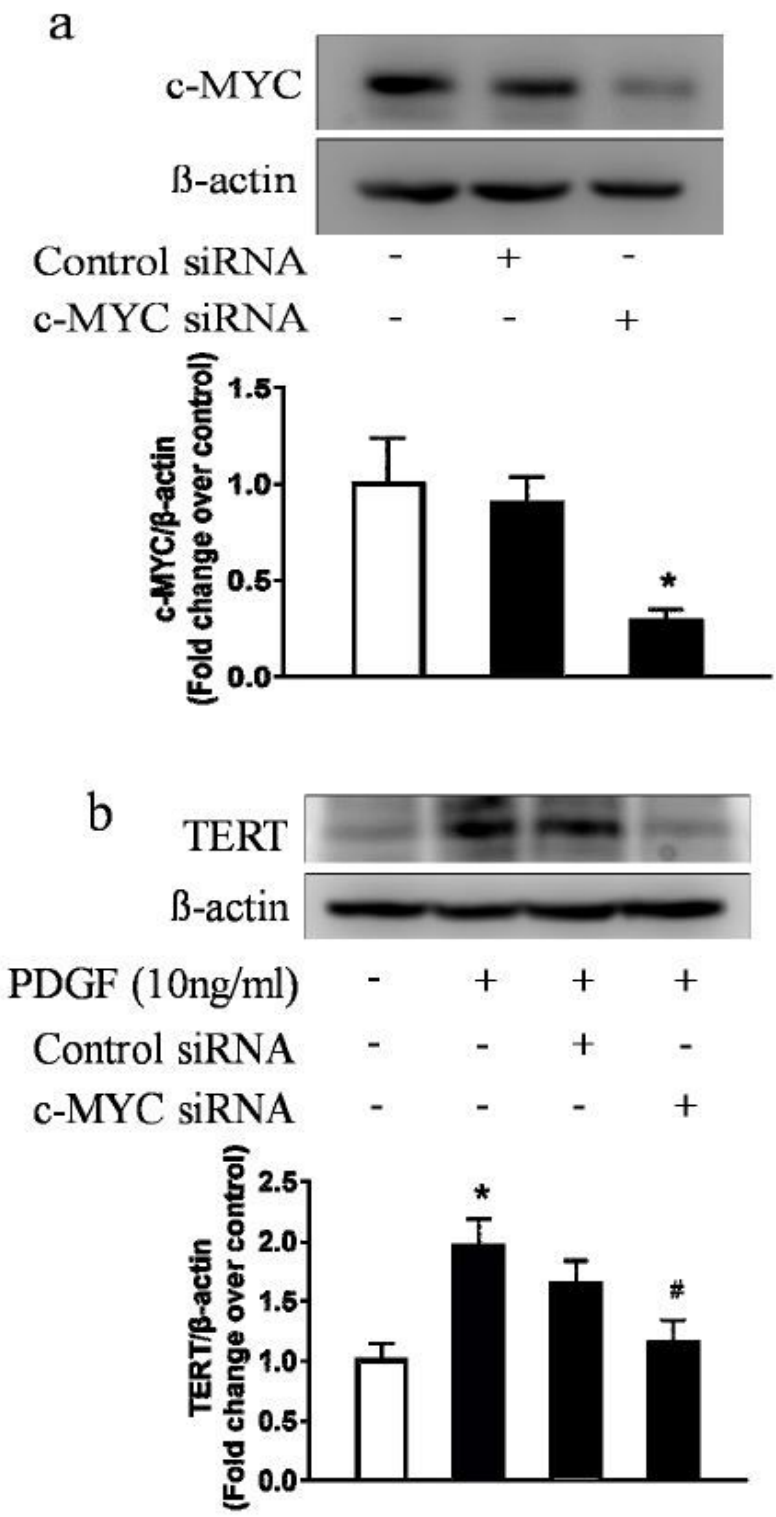

Figure 4

c-MYC mediates the expression of TERT in PDGF-induced PASMCs proliferation. (a) c-MYC siRNA or non-targeting siRNA were transfected into cells, the silencing effect was detected by western blotting. β-actin served as loading control. (n=3 per group). * $P < 0.05$ versus control. (b) c-MYC siRNA or non-targeting siRNA were prior transfected to cells and then treated with PDGF for 24 h. TERT protein level was

measured using western blotting. β- actin served as loading control (n=4 per group). * P < 0.05 versus control. #P < 0.05 versus PDGF-treated group.

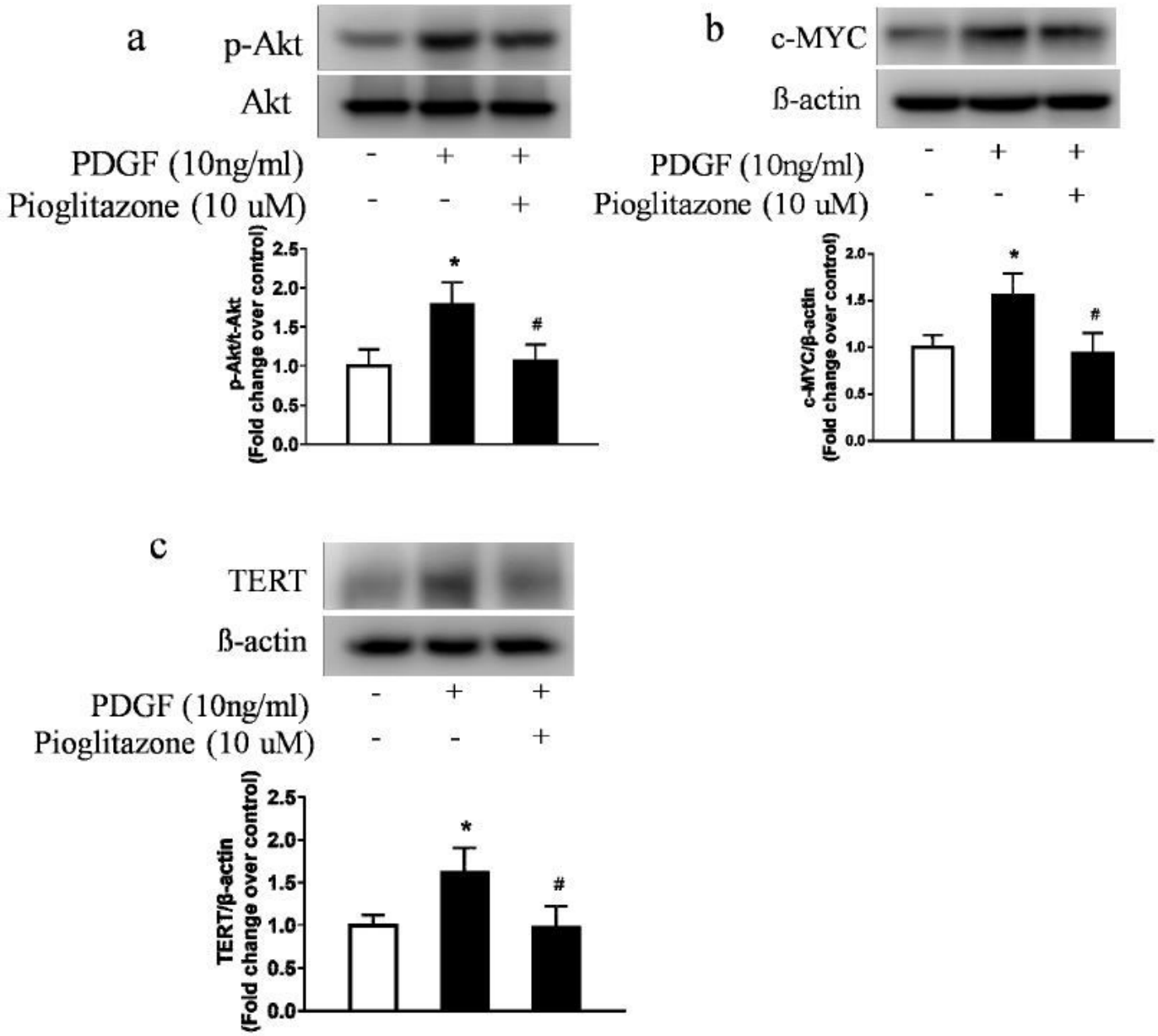

Figure 5

Activation of PPARγ inhibits Akt/c-MYC/TERT signal pathway in PASMCs. Pioglitazone (10 mM) pre-treated PASMCs for 30 min before PDGF stimulation. (a) Phosphorylation of Akt was determined by western blotting (n=3 per group). (b) the expression of c-MYC was detected using western blotting (n=3 per group). (c) TERT protein level was measured by western blotting (n=3 per group). *P < 0.05 versus control, #P < 0.05 versus PDGF -treated cells.

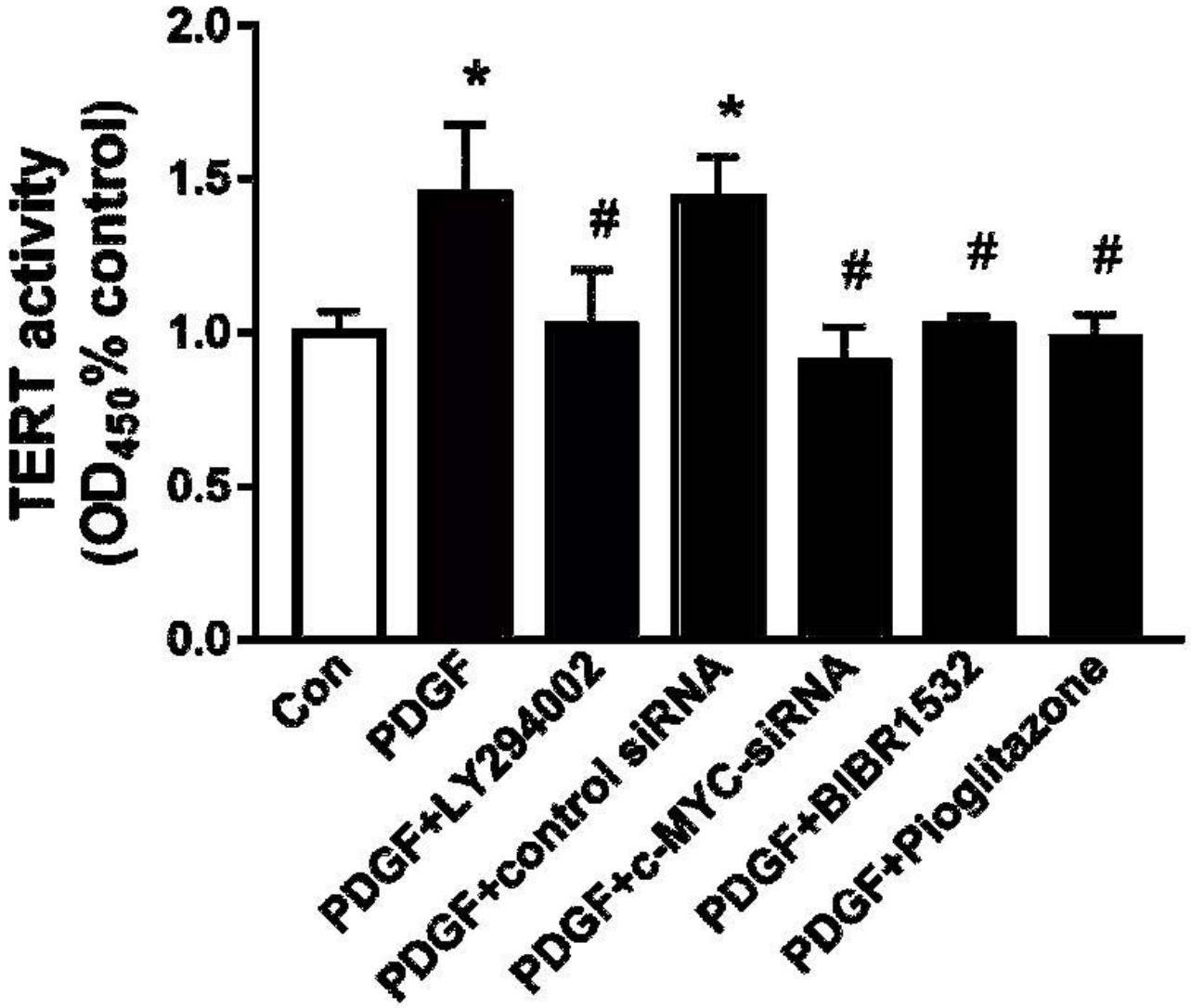

Figure 6

Activation of PPARγ suppresses PDGF-induced telomerase activation in PASMCs. Cells were pre-incubated with pioglitazone (10 mM) for 30 min, LY294002 for 30 min, BIBR1532 for 2 h, prior transfected with non-targeting siRNA or c-MYC siRNA for 24 before PDGF stimulation 10 ng/ml for 24 h. telomerase activity was measured using Rat TE ELISA KIT (n=6 per group). * $P < 0.05$ versus control, #$P < 0.05$ versus PDGF -treated cells.

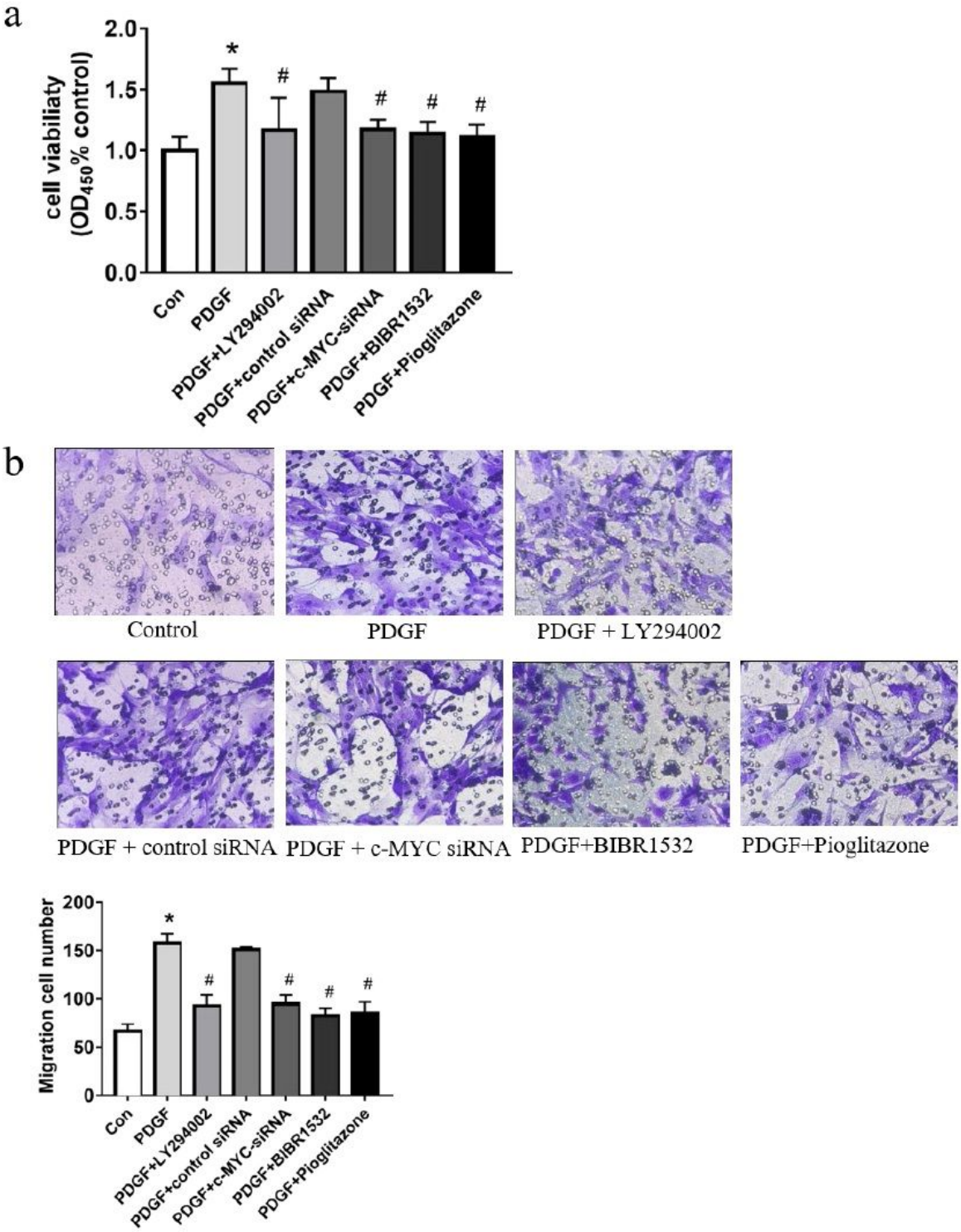

Figure 7

PDGF induces PASMCs proliferation and migration via Akt/c-MYC/TERT axis. Cells were pre-incubated with pioglitazone (10 mM) for 30 min, LY294002 for 30 min, BIBR1532 for 2 h, prior transfected with non-targeting siRNA or c-MYC siRNA for 24 before PDGF stimulation 10 ng/ml for 24 h. (a) The proliferation of PASMCs was detected through CCK-8 (n=6 per group). (b) The migration of PASMCs was examined by transwell assay (n=3 per group). $P < 0.05$ versus control, #$P < 0.05$ versus PDGF -treated cells.