Metabolic dependence of cyclosporine A on cell proliferation of human non-small cell lung cancer A549 cells and its implication in post-transplant malignancy

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Abstract. Cyclosporine A (CsA), a widely used immunosuppressant to prevent organ transplant rejection, is associated with an increased cancer risk following transplantation, particularly in the lung. However, the underlying mechanisms remain unclear. In the present study, using human non-small cell lung cancer A549 cells, it was determined that CsA (0.1 or 1 μM) promoted cell proliferation with glucose alone as the energy source. CsA treatment increased the phosphorylation of protein kinase B (Akt) and consequently the expression of Cyclin D1. Inhibiting Akt signaling with the phosphatidylinositol 3-kinase inhibitor wortmannin prevented this effect. Mechanistically, CsA treatment increased reactive oxygen species (ROS) generation, and the intracellular ROS scavenger N-acetyl-cysteine (NAC) attenuated CsA-induced cell proliferation as well as the activation of Akt/Cyclin D1 signaling. However, notably, it was demonstrated that CsA treatment decreased cell proliferation and Akt phosphorylation under normal lipid loading. Further investigation indicated that palmitic acid induced excessive generation of ROS, while CsA treatment further stimulated this ROS production. Scavenging intracellular ROS with NAC attenuated the CsA-mediated inhibition of cell proliferation. Collectively, the results indicated a pleiotropic effect of CsA in the regulation of A549 cell proliferation under different metabolic conditions. This indicated that CsA administration may contribute to increased post-transplant cancer risk in organ recipients.

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Abbreviations: CsA, cyclosporine A; BMI, body mass index; PA, palmitic acid; Wm, Wortmannin; ROS, reactive oxygen species

Key words: cyclosporine A, lung cancer, metabolic substrates, protein kinase B, reactive oxygen species

Introduction

Organ transplantation is not only considered a last-resort life-saving therapy, but also as the standard treatment of choice for numerous patients with end-stage organ damage (1). However, patients undergoing transplantation may suffer from various complications, including cancer, infection and cardiovascular disease (2). Among them, malignancy development following organ transplantation has become a more pressing issue in the past decade (3-5), as mortalities as a result of cardiovascular disease and infection decrease in frequency with the advancement of medical techniques (6). A three- to four-fold increased risk of cancer has been observed in transplant patients in USA in 2011, compared with the age-matched general population (3). However, the detailed mechanism of post-transplant malignancy remains poorly understood.

Organ recipients administer immunosuppressive drugs to prevent the body rejecting the organ. Cyclosporine A (CsA), an inhibitor of calcineurin, is frequently used as an immunosuppressive drug to prevent organ transplant rejection (7). It has been well-documented that immunosuppressant therapy increases the risk of post-transplant cancer (4,8,9). Hojo *et al* (10) demonstrated a tumor growth-promoting effect of CsA in immunodeficient mice, which was attributed to transforming growth factor-β upregulation. However, Sato *et al* (11) reported that CsA at high concentrations (1 μg/ml) induces apoptosis in human lung adenocarcinoma cells in a caspase-dependent manner. Thus, the effect of CsA on cell proliferation requires further investigation.

Additionally, increasing evidence demonstrated that metabolic homeostasis is crucial in maintaining human health (12). Obesity is associated with metabolic alterations and is considered an important risk factor for the development of a number of cancer types, including colon, breast, kidney and lung cancer (6,8-11). However, in patients with cardiovascular disease, chronic renal failure, chronic pulmonary obstructive disease, acquired immune deficiency syndrome or

rheumatoid arthritis, the presence of obesity appears to be a paradoxical protective factor for their survival (13,14), which is termed the 'obesity paradox', and has been confirmed by clinical investigation (15,16). In line with these observations, the obesity paradox was also demonstrated to occur in organ recipients (17). As increased free fatty acids are detected in overweight and obese subjects (18), the metabolic dependence of immunosuppressants on carcinogenesis requires further studies, as it may account for this paradox.

Lung cancer is among the four most common cancer types in transplant recipients in USA in 2011, particularly following lung transplantation (4,19-21). Therefore, in the present study, the effect of CsA on carcinogenesis in human non-small cell lung cancer A549 cells exposed to different metabolites (glucose or palmitic acid) was examined, and the underlying mechanisms were determined.

Materials and methods

Cell culture. The human non-small cell lung cancer cell line A549 was obtained from Shanghai Meixuan Biological Technology Co., Ltd. (Shanghai, China). A549 cells were cultured in a 37˚C humidified incubator with 5% CO_2 in RMPI-1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA); and 1% penicillin/streptomycin (100X; Invitrogen; Thermo Fisher Scientific, Inc.). Medium was replaced every 2 days.

Reagents. Reagents included: CsA (Medchem Express, Princeton, NJ, USA); wortmannin (Wm; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); RMPI-1640; penicillin/streptomycin(100X);FBS;N-acetyl-cysteine(NAC;Invitrogen;Thermo Fisher Scientific, Inc.); Palmitic acid (PA; Sigma-Aldrich; Merck KGaA); D-Arg-2',6'-dimethyltyrosine-Lys-Phe-NH2 (SS-31; supplied by Dr Xing Zhang from Department of Aerospace Medicine, Fourth Military Medical University, Xi'an, China).

Cell viability assay. Cell viability was measured using a commercial Cell Counting Kit-8 from Medchem Express (cat. no. HY-K0301), according to manufacturer's protocol. The cytotoxic effect of CsA against A549 cells under glucose loading were examined as aforementioned at the concentrations of 0, 0.1 and 1 μM at 37˚C for 48 h in RMPI-1640 medium. To determine the effect of glucose in CsA-induced A549 cell proliferation, 5, 10, 20 and 30 mM glucose was replaced with equimolar mannitol, the isotonic control of glucose. To determine the role of phosphoinositide 3-kinase (PI3K)/Akt signaling in CsA-induced cell proliferation, A549 cells were treated with PI3K inhibitor Wm (200 nM) prior to CsA treatment, and cell viability was assessed as aforementioned. The cytotoxic effect of PA against A549 cells were examined as above mentioned for 48 h in RMPI-1640 medium supplemented with 0, 50, 100, 200 or 500 μM PA. The cytotoxic effect of CsA against A549 cells under normal lipid loading were examined as aforementioned at 0.1 μM (the optimal dose for cell proliferation) for 48 h in RMPI-1640 medium supplemented with 200 μM PA. To determine the role of ROS in CsA-induced A549 cell proliferation, intracellular ROS scavenger (NAC; 10 μM) or mitochondrial ROS scavenger (SS-31; 10 μM) were added to the culture prior to CsA treatment, and cell viability was assessed as aforementioned.

EdU incorporation assay. A549 cells were cultured in 35 mm confocal dishes and treated with or without CsA (1 μM) at 37˚C for 48 h. All cells were treated with 50 μM EdU for 2 h at 37˚C, and fixed with 4% paraformaldehyde for at room temperature 15 min. The fixed cells were treated with 0.3% Triton X-100 at room temperature for 10 min and washed with PBS three times. Thereafter, the cells were exposed to Click reaction solution (Beyotime Institute of Biotechnology, Haimen, China) for 30 min, followed by incubation with 5 μM Hoechst 33342 at room temperature for 10 min to stain the cell nuclei. Images were captured using an inverted confocal microscope (Zeiss LSM 800; Carl Zeiss AG, Oberkochen, Germany) with a x40 1.3NA oil-immersion objective. The proliferation index was calculated by dividing the number of EdU-labeled cells by the total number of cells (Hoechst-positive).

PA preparation. Stock PA was dissolved in ethanol at a concentration of 10 mM and diluted to 50, 100, 200 or 500 μM in RMPI-1640 containing 1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA). As a vehicle control, the same volume of ethanol as used in the 50 μM PA group was diluted in RMPI-1640 containing 1% (w/v) BSA.

Determination of intracellular reactive oxygen species (ROS) production. Intracellular ROS production was measured using a dihydroethidium (DHE) probe. Briefly, A549 cells were treated with or without 0.1 μM CsA under normal glucose or lipid loading at 37˚C for 48 h, and incubated with 2.5 μM DHE for 30 min at 37˚C. After cells were washed in PBS three times, fluorescence was measured with a FluoStar Omega (BMG Labtech GmbH, Ortenberg, Germany) at excitation and emission wavelengths of 480 and 590 nm, respectively.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from treated A549 cells with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols, and reverse transcribed into cDNA with the PrimeScript RT Reagent kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan). Subsequently, qPCR was performed using SYBR® Premix Ex Taq II (Takara Bio, Inc.) on a CFX96 real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermocycling conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec, with a final extension at 72˚C for 10 min. Relative mRNA expression levels of Cyclin D1 and p27 were quantified using the $2^{-\Delta\Delta Cq}$ method (22), and the results were normalized to β-actin as an internal control. The sequences of primer sets used in this analysis were as follows: Cyclin D1, forward, 5'-TGTCCT ACTACCGCCTCACA-3', and reverse, 5'-CAGGGCTTCGAT CTGCTC-3'; p27, forward, 5'-TAATTGGGGCTCCGGCTA ACT-3', and reverse, 5'-TGCAGGTCGCTTCCTTATTCC-3'; and β-actin, forward, 5'-CGCCCCAGGCACCAGGGC-3', and reverse, 5'-GCTGGGGTGTTGAAGGT-3'.

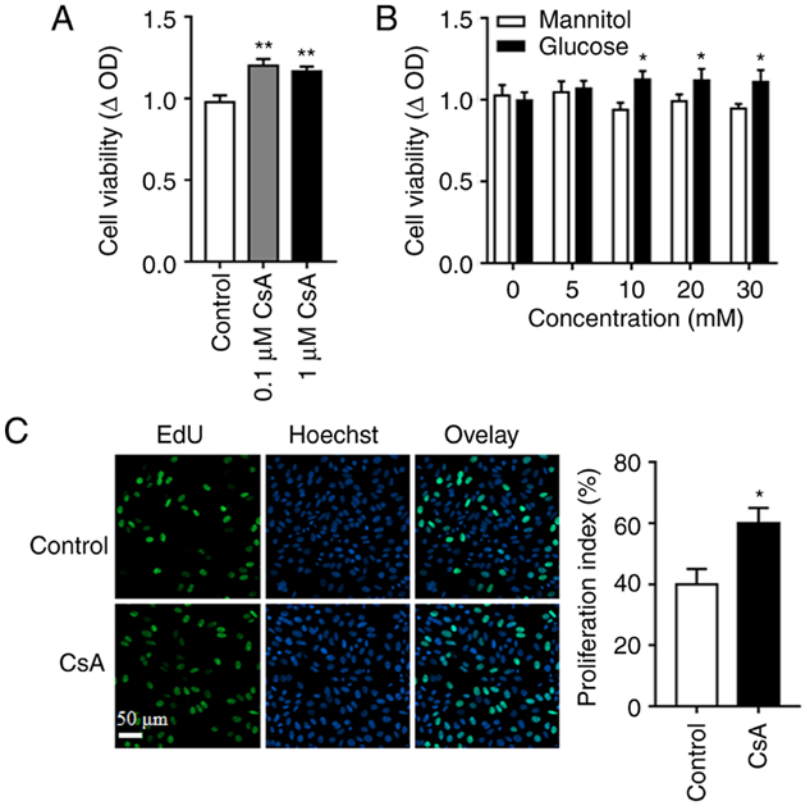

Figure 1. CsA promotes cell proliferation in A549 cells under glucose loading. (A) Cell proliferation in response to different concentrations of CsA (0, 0.1 and 1 μM) for 48 h was assessed with a CCK-8 assay. (B) Cell proliferation under different concentrations of glucose (0, 5, 10, 20 and 30 mM) was assessed with CCK-8 assays. (C) Quantitative analysis of EdU-labeled cell distribution in the absence or presence of 1 μM CsA treatment. All values are expressed as the mean ± standard error of the mean of three independent experiments. *P<0.05 vs. 0 mM glucose group or control; **P<0.01 vs. control. CsA, cyclosporine A; CCK, Cell Counting Kit; OD, optical density.

Western blotting. Following drug treatment, A549 cells were washed with cold PBS three times and lysed in radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were incubated on ice for 15 min, and then cleared by high-speed centrifugation (13,000 x g at 4˚C for 15 min). Subsequently, the total protein concentration was measured with a bicinchoninic acid protein assay. Protein samples were separated by SDS-PAGE (15%), and then transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% milk at room temperature for 1 h and subsequently incubated with the appropriate primary antibodies against Cyclin D1 (cat. no. AF1183; 1:5,000; 34 kDa; Beyotime Institute of Biotechnology), p27 (cat. no. AP027; 1:5,000; 27 kDa; Beyotime Institute of Biotechnology), caspase-3 (cat. no. 9662; 1:1,000; 35 kDa; Cell Signaling Technology, Inc., Danvers, MA, USA), cleaved caspase-3 (cat. no. 9664; 1:1,000; 17 kDa; Cell Signaling Technology, Inc.), phospho-protein kinase B (cat. no. 4060; 1:1,000; Akt; S473; Cell Signaling Technology, Inc.), Akt (cat. no. 4691; 1:1,000; 60 kDa; Cell Signaling Technology, Inc.) and β-actin (cat. no. AF0003; 1:1,000; 43 kDa; Beyotime Institute of Biotechnology) at 4˚C overnight. Subsequently, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (cat. no. A0208; 1:1,000; Beyotime Institute of Biotechnology) or goat anti-mouse IgG antibody (cat. no. A0216; 1:1,000; Beyotime Institute of Biotechnology) for 1-2 h at room temperature. Finally, the blots were visualized using an Enhanced Chemiluminescence-Plus reagent (Millipore, Billerica, MA, USA), and detected by ChemiDocXRS (Bio-Rad Laboratories, Inc.) and analyzed with a Bio-Image Analysis system (Bio-Rad Laboratories, Inc.).

Statistical analysis. Student's unpaired t-test or one-way analysis of variance followed by Bonferroni's post hoc test were used for statistical analysis of cellular data. Data are shown as the mean ± standard error of the mean. Statistical tests were performed using GraphPad Prism software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Cyclosporine A promotes cell proliferation in A549 cells under glucose loading. To investigate the effects of CsA on post-transplant malignancy, A549 cell proliferation in response to different concentrations of CsA (0, 0.1 and 1 μM) for 48 h was measured, and the results demonstrated the pro-cancer effect of CsA (Fig. 1A). Furthermore, by replacing glucose with mannitol, the isotonic control of glucose, it was indicated that CsA promoted cell proliferation when glucose concentration was high (10, 20 and 30 mM; Fig. 1B), demonstrating that glucose is a vital factor in CsA-induced cell proliferation. In line with these results, the number of EdU-labeled cells following CsA treatment increased, compared with the control group (Fig. 1C).

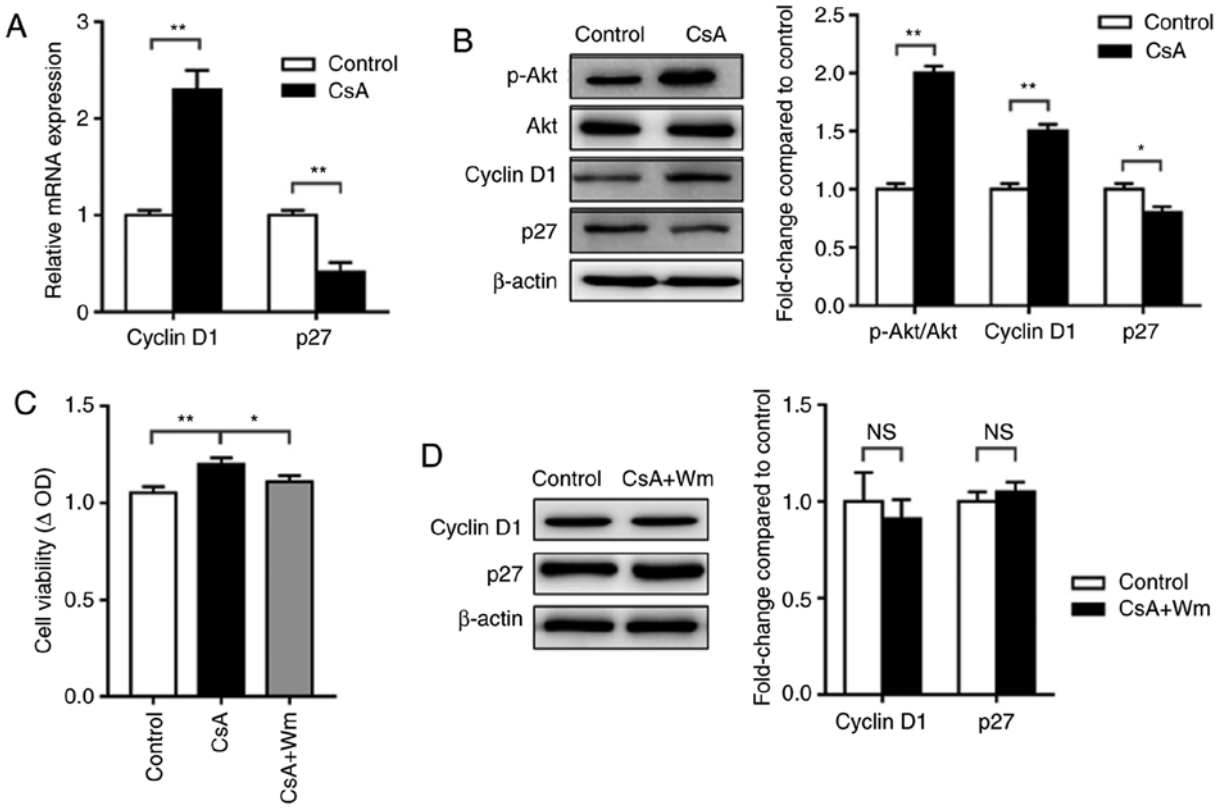

Figure 2. Activation of PI3K/Akt signaling contributes to CsA-induced cell proliferation. (A) Reverse-transcription quantitative polymerase chain reaction analysis of Cyclin D1 and p27 mRNA in the absence or presence of CsA treatment (0.1 μM). (B) Western blot analysis of cell cycle-associated proteins (Akt, Cyclin D1 and p27) in the absence or presence of CsA treatment (0.1 μM). (C) A549 cell proliferation was assessed following pharmacological intervention of PI3K/Akt signaling with Wm (200 nM) prior to CsA treatment (0.1 μM). (D) Western blot analysis of cell cycle-associated proteins (Cyclin D1 and p27) in control or CsA+Wm groups. All values are expressed as the mean ± standard error of the mean of three independent experiments. *P<0.05 and **P<0.01. NS, no significance; CsA, cyclosporine A; Akt, protein kinase B; PI3K, phosphatidylinositol 3-kinase; Wm, wortmannin.

Involvement of PI3K/Akt signaling pathway in CsA-induced cell proliferation. It is well-documented that aberrant Akt activation contributes to lung carcinogenesis (23,24), and PI3K/Akt signaling is involved in the regulation of various cell functions, including cell survival, proliferation and cell cycle progression (25). RT-qPCR analysis of the expression of cell cycle-associated genes revealed that 0.1 μM CsA increased Cyclin D1 mRNA expression and decreased p27 mRNA expression (Fig. 2A). Similarly, western blot analysis demonstrated that CsA increased the phosphorylation of Akt and the expression of Cyclin D1, while decreasing the expression of p27 (Fig. 2B). Pharmacological intervention of PI3K/Akt signaling with Wm attenuated CsA-induced cell proliferation (Fig. 2C), while slightly increasing the expression of p27 and decreasing the expression of Cyclin D1 (Fig. 2D). These results indicated the involvement of PI3K/Akt signaling in CsA-induced cell proliferation.

Intracellular ROS scavenger NAC attenuates CsA-induced cell proliferation. ROS-mediated activation of Akt has been well-documented (26), and our previous study demonstrated that CsA increases intracellular ROS production in insulin-resistant C2C12 cells (27). In line with this, the present study indicated that CsA treatment increased intracellular ROS production in A549 cells (Fig. 3A). Additionally, NAC attenuated CsA-induced cell proliferation, whereas SS-31, an efficient mitochondrion-targeted antioxidant, did not significantly affect this process (Fig. 3B), which may be due to the predominance of glycolysis, instead of the Krebs cycle, in cancer cells (28). Furthermore, intracellular ROS scavenger NAC decreased CsA-mediated Akt activation as well as Cyclin D1 expression, while increasing p27 expression (Fig. 3C). These results indicated that ROS-mediated activation of Akt contributed to CsA-induced cell proliferation in A549 cells under normal glucose loading.

CsA decreases cell proliferation under normal lipid loading. Obesity, characterized as the alteration of metabolic balance between glucose and fatty acid oxidation, is associated with reduced mortality, termed the 'obesity paradox' (29). Thus, the effect of fatty acids on CsA-mediated cell proliferation was investigated. PA is the most prevalent saturated free fatty acid (FFA) in circulation, accounting for ~28% of FFAs in serum (30). The effect of different concentrations of PA on cell viability was assayed, and the results demonstrated that 200 or 500 μM PA significantly decreased A549 cell proliferation (Fig. 4A). Notably, under normal lipid loading (200 μM PA), CsA decreased A549 cell proliferation (Fig. 4B), indicating a divergent role of CsA on cell proliferation in the presence of different metabolic substrates. In line with this observation, a decrease in cell proliferation by CsA was accompanied by decreased Akt phosphorylation and increased cleaved caspase-3 expression (Fig. 4C).

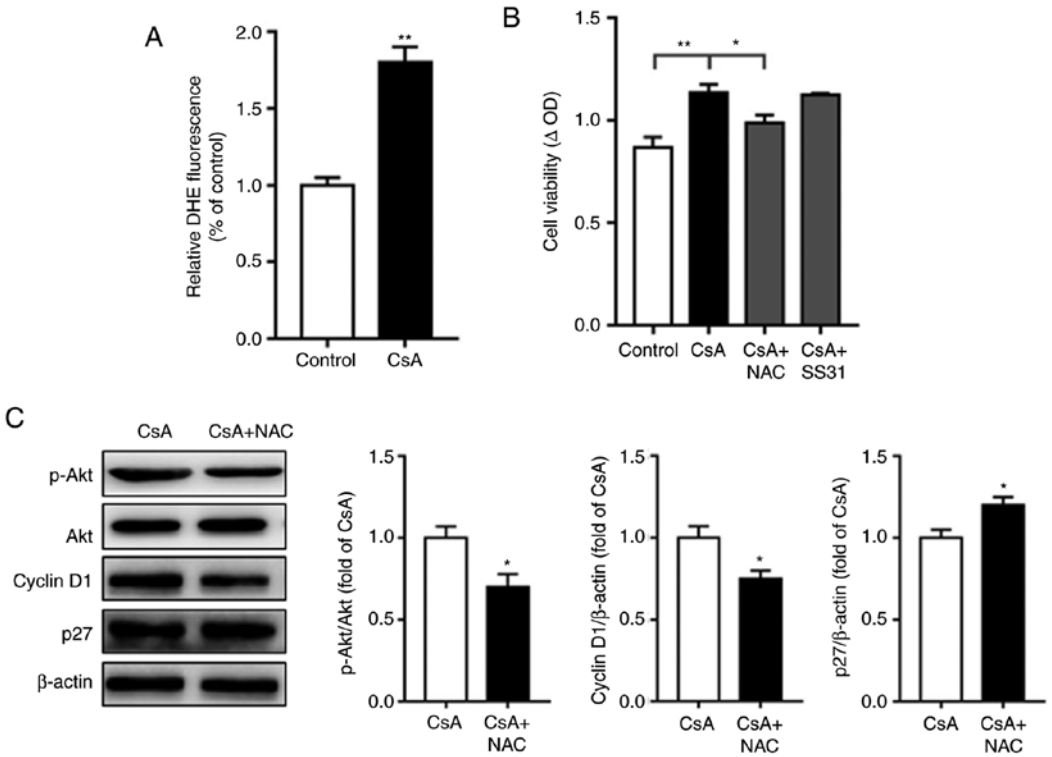

Figure 3. Intracellular ROS scavenger NAC attenuates CsA-promoted cell proliferation. (A) Intracellular ROS production was measured using a DHE probe. (B) Cell proliferation was assessed in A549 cells when incubating with NAC (intracellular ROS scavenger) or SS31 (mitochondrial ROS scavenger) prior to CsA treatment. (C) Western blot analysis of cell cycle-associated proteins in A549 cells with NAC pre-incubation prior to CsA treatment. All values are expressed as the mean ± standard error of the mean of three independent experiments. *P<0.05 vs. CsA group; **P<0.01 vs. control. DHE, dihydroethidine; CsA, cyclosporine A; ROS, reactive oxygen species; OD, optical density; p-Akt, phospho-protein kinase B; NAC, N-acetyl-cysteine; SS-31, D-Arg-2',6'-dimethyltyrosine-Lys-Phe-NH2.

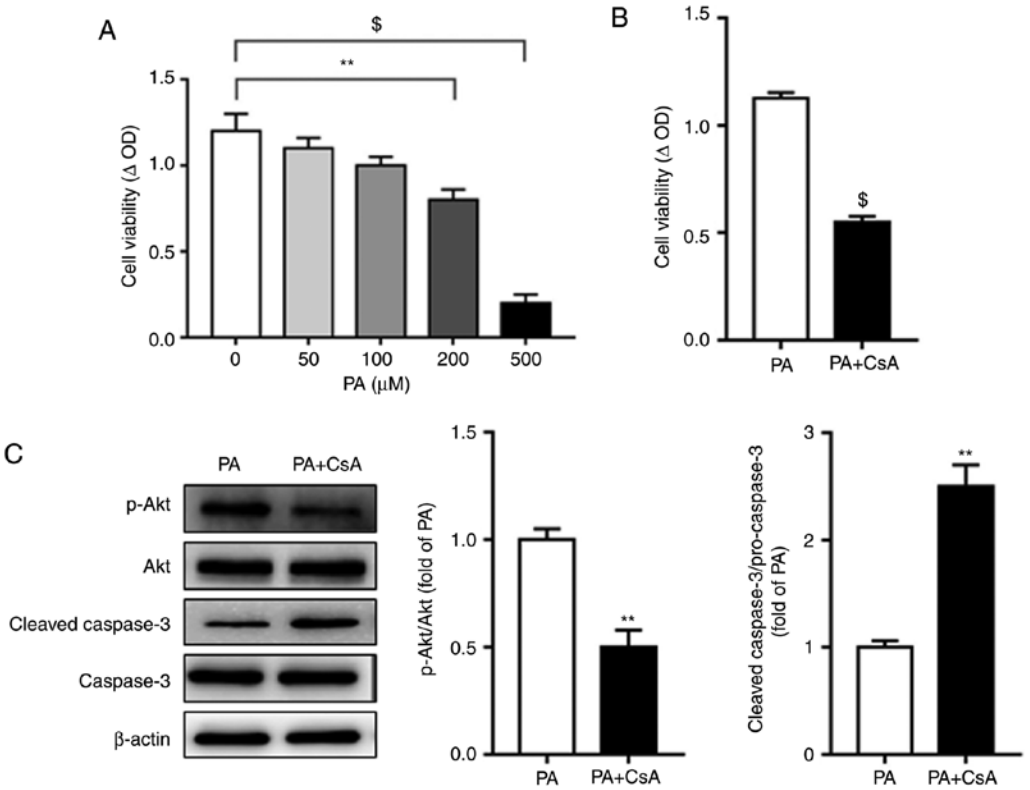

Figure 4. CsA decreases cell proliferation under normal lipid loading. (A) Cell proliferation was assessed in the presence of different concentrations of PA (0, 50, 100, 200 and 500 μM) in A549 cells cultured in RPMI-1640 medium. (B) Cell proliferation was assessed in the absence or presence of 0.1 μM CsA in A549 cells cultured in RPMI-1640 medium supplemented with 200 μM PA. (C) Western blot analysis of survival and apoptotic signaling in A549 cells treated with or without CsA under normal lipid loading (100 μM PA). All values are expressed as the mean ± standard error of the mean of three independent experiments. **P<0.01 and $P<0.0001 vs. 0 μM PA group. PA, palmitic acid; CsA, cyclosporine A; OD, optical density; p-Akt, phospho-protein kinase B.

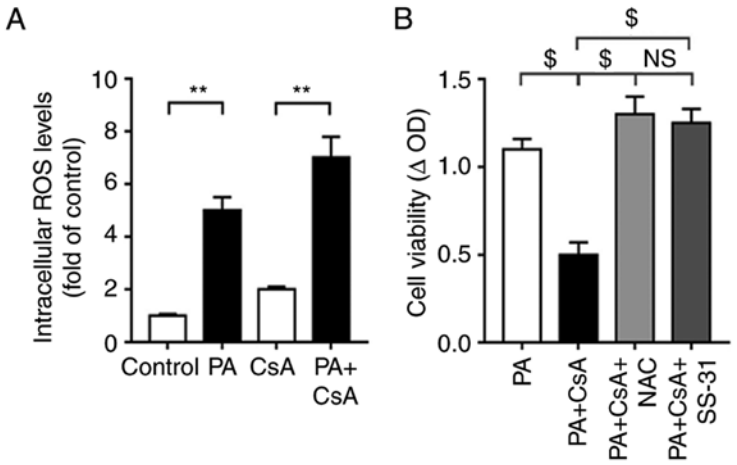

Figure 5. Excessive ROS contributes to CsA-mediated inhibition of cell proliferation under normal lipid loading. (A) Following treatment with control, CsA, PA and PA+CsA, intracellular ROS production was measured using a dihydroethidine probe. (B) Cell proliferation was assessed in A549 cells when incubating NAC (intracellular ROS scavenger) or SS-31 (mitochondrial ROS scavenger) prior to CsA treatment under normal lipid loading. All values are expressed as the mean ± standard error of the mean of three independent experiments. **P<0.01 and $^{\$}$P<0.0001. NS, no significance; CsA, cyclosporine A; ROS, reactive oxygen species; NAC, N-acetyl-cysteine; SS-31, D-Arg-2',6'-dimethyltyrosine-Lys-Phe-NH2; PA, palmitic acid; CsA, cyclosporine A.

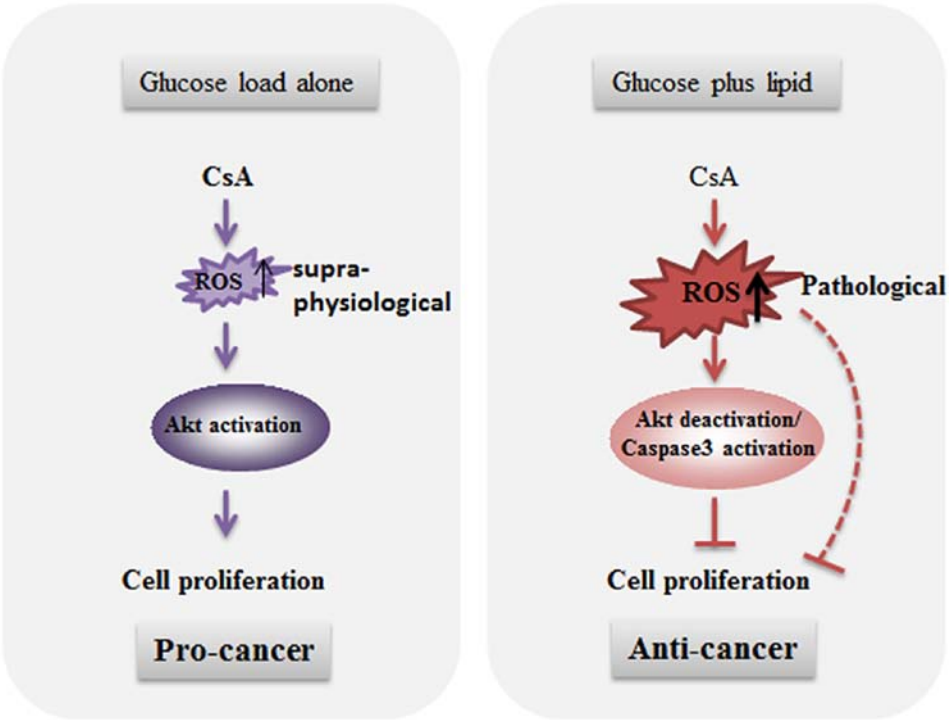

Figure 6. Schematic illustrating the pleiotropic effect of CsA in the regulation of A549 cell proliferation under different metabolic conditions. CsA (0.1 or 1 μM) promoted A549 cell proliferation with glucose load alone, which was ascribed to moderate ROS generation and resultant activation of Akt/Cyclin D1 signaling. However, under normal glucose plus lipid loading, 0.1 μM CsA stimulated excessive ROS production, and exerted anti-cancer effect. CsA, cyclosporine A; Akt, protein kinase B; ROS, reactive oxygen species.

Excessive ROS contributes to CsA-mediated inhibition of cell proliferation under normal lipid loading. To investigate underlying mechanisms, intracellular ROS levels were assessed using a DHE probe. The results demonstrated that compared with the control group (glucose alone as the energy source), PA significantly increased intracellular ROS levels (Fig. 5A). Scavenging intracellular ROS with NAC, or mitochondrial ROS with SS-31, attenuated the CsA-mediated A549 cell proliferation inhibition under PA load, while no significant changes in cell viability were observed between the PA+CsA+NAC and PA+CsA+SS-31 groups (Fig. 5B). These results demonstrated the divergent roles of CsA on cell proliferation in the presence of different metabolic substrates.

Discussion

Increasing evidence demonstrated that organ transplantation is associated with an increased risk of (3-5). Recently, the immunosuppressor CsA was demonstrated to contribute to post-transplant malignancy (8,9). However, the effects of

CsA on cell proliferation were unclear and required further investigation. In the present study, the pleiotropic effects of CsA on carcinogenesis in the presence of different metabolic substrates (glucose or PA) were reported. When cultured under glucose loading, CsA increased cell proliferation as well as Akt/Cyclin D1 signaling; however, this pro-cancer effect was reversed when supplemented with 200 μM PA.

Although malignancy accounts for a small percentage of mortality in the first year after transplantation, the International Society for Heart and Lung Transplantation Registry reports in 2012 that malignancy accounts for ~15% of mortalities beyond 5 years post-transplantation based on data from centers globally (4,5). Non-melanoma skin cancer and post-transplant lymphoproliferative disease are the most common post-transplant malignancies (5). However, lung cancer, including non-small cell and small cell lung carcinoma, is increasingly becoming a frequent complication in patients (31). Genetic, cellular, molecular and environmental factors all serve a crucial role in post-transplant carcinogenesis (4). Previous studies demonstrated that tumor incidence increases with time following organ transplantation and is associated with the intensity of immunosuppression (32,33). Certain studies indicated that CsA treatment inhibits carcinogenesis (34,35), whereas others reported contradictory results (11,36). Thus, further investigation of CsA on carcinogenesis is of critical importance.

Increasing studies demonstrated that among patients with cancer, elevated body mass index (BMI) is associated with improved survival, compared with normal-weight patients, indicating the existence of an 'obesity paradox' (29). Furthermore, a paradoxical U-shaped association of BMI with outcomes is also observed in transplant recipients (37,38). Obesity is characterized by metabolic abnormalities, including hyperglycemia, insulin resistance and hyperlipidemia; thus, alterations of metabolic substrate preference may be of critical importance in this paradox (39). The efficient use of CsA as an immunosuppressant has been limited by its toxic effects, including nephrotoxicity, hepatotoxicity and neurotoxicity. Additionally, obese patients have an increased risk of toxicity, compared with lean subjects, and a smaller dose is required for obese transplantation recipients (40), indicating that alterations of metabolic substrates may contribute to differential toxicities of CsA in obese and lean subjects. In the present study, the pleiotropic effects of CsA in the regulation of cell proliferation in human lung adenocarcinoma cells exposed to different metabolic substrates was reported.

An association between ROS and CsA-induced toxicity has been reported (41). The results of the present study, as well as previous studies, demonstrated that CsA treatment induces ROS production and lipid peroxidation (27,42,43). It has been well established that supra-physiological levels of ROS may activate/deactivate certain signaling molecules, such as Akt, and affect a number of physiological processes, including regulation of cell cycle, cell proliferation and survival (26). Furthermore, pathological levels of ROS have an important role in apoptosis induction. Therefore, the results demonstrated that supra-physiological levels of ROS induced by CsA promoted Akt signaling and cell proliferation under glucose loading, whereas under glucose/PA loading, CsA treatment induced pathological levels of ROS production and decreased cell viability.

Collectively, the data indicated a divergent role of CsA in the regulation of A549 cell proliferation with different metabolic substrates (Fig. 6), indicating that the CsA-mediated increase in cell proliferation could contribute to increased post-transplant cancer risk.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZC designed the research, analyzed the results and revised the manuscript. XQ performed the experiments, interpreted the results of experiments and drafted the manuscript. Both authors approved the final version of manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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