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AMP-activated protein kinase (AMPK) activation is involved in chrysin-induced growth inhibition and apoptosis in cultured A549 lung cancer cells

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ABSTRACT

Here we show that chrysin induces growth inhibition and apoptosis in cultured lung cancer A549 cells, and activation of AMP-activated protein kinase (AMPK) may contribute to this process. Our Western-blots results demonstrated a significant AMPK activation after chrysin treatment in A549 cells. Inhibition of AMPK by shRNA-mediated gene silencing, or by its inhibitor, diminished chrysin-induced A549 cell growth inhibition and apoptosis. Forced activation of AMPK by introducing a constitutively active form of AMPKα (CA-AMPKα), or by its activators, mimicked chrysin's effect. For mechanism analysis, we found chrysin inhibited Akt/mammalian target of rapamycin (mTOR) activation, and knocking-down of AMPK by shRNA almost reversed this effect. Finally, we observed that a relative low dose of chrysin enhanced doxorubicin-induced AMPK activation to promote A549 cell apoptosis. Our study suggests that activation of AMPK by chrysin contributes to Akt suppression, growth inhibition and apoptosis in human lung cancer cells, and agents that could activate AMPK may serve as useful adjuvants for traditional chemotherapy against lung cancer.

1. Introduction

Lung cancer is one of the leading causes of cancer-related death. In the United States, approximately one third of all cancer-related deaths are due to this disease [1]. Despite the tremendous improvements in diagnosis and treatments, the overall 5 year survival rate remains below 15% [2]. Resistance to chemotherapy and radiotherapy has been considered as the major obstacle to successful treatments, and the search for new adjuvants is urgent.

Chrysin (5,7-dihydroxyflavone) is a natural flavonoid that is widely distributed in medicinal herbs [3,4]. Different groups have confirmed the anti-growth properties of chrysin against human cancer cells [5–8]. However, the potential anti-tumorigenic effects of chrysin in lung cancer cells and the potential mechanisms of action remain largely unknown.

Recent studies have confirmed that AMP-activated protein kinase (AMPK), the master energy sensor, is also the key regulator of cell apoptosis under pathological stress conditions [9–11]. Activation of AMPK promotes cell apoptosis through regulating its downstream signaling targets, which include JNK [12–14], p53 [15,16] and mammalian target of rapamycin (mTOR) [7,17,18]. In this study, we investigated the potential role of AMPK in chrysin-treated A549 lung cancer cells.

2. Material and methods

2.1. Antibodies and reagents

Chrysin, doxorubicin, N-acetyl-cysteine (NAC), and monoclonal mouse anti-β-actin antibody were obtained from Sigma (Shanghai, China). AMPKα, p-AMPKα (Thr 172), AMPKβ, p-ACC (Ser 79), ACC, p-Akt (Ser 473, Thr 308), p-S6 (Ser 235/236), p-4E-BP1 (Ser 65), Akt1 and S6 antibodies were obtained from Cell Signaling Technology (Shanghai, China). Rabbit IgG-HRP and mouse IgG-HRP antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Compound C, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), A-769662 and Z-DEVD-fmk were purchased from CalbioChem (Shanghai, China).

2.2. Cell culture

The human lung cancer cell line A549 was obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured in DMEM medium supplemented with

Abbreviations: AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; CI, combination index; FACS, fluorescence-activated cell sorting; PI, propidium iodide; JNK, c-Jun N-terminal kinase; mTOR, mammalian target of rapamycin; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl-cysteine; ROS, reactive oxygen species.

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10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/L) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Cell viability assay (MTT assay)

Cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) method. Briefly, A549 cells were seeded in 96-well plates at a density of 3×10^5 cells/ml. After indicated treatments, MTT tetrazolium salt (0.25 mg/ml) was added to each well and incubated in CO_2 incubator for 4 h at 37 °C, 150 μl of DMSO was then added to dissolve formazan crystals and the absorbance of each well was observed by a plate reader at a test wavelength of 490 nm.

2.4. Western-blots

Cells were washed with ice-cold PBS, scraped into PBS, and collected by centrifugation. Pellets were re-suspended in a lysis buffer containing 50 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10% glycerol, 0.5% NP-40, 0.5% Tween 20, 1 mmol/L dithiothreitol, and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN); insoluble material was removed by centrifugation. Protein concentration was measured. Proteins (40 μg) were resolved by SDS–PAGE and transferred to PVDF membranes. Membranes were incubated sequentially in TBS containing 0.05% Tween-20 and 5% nonfat dry milk as follows: no addition, 1 h at room temperature (blocking); primary antibody, overnight at 4 °C; and secondary antibody (Amersham), 2 h at room temperature. Bound secondary antibody was detected by ECL system. Western blot results were quantified by Image J software from NIH website.

2.5. Caspase-3 activity assay

After indicated treatment(s), cytosolic proteins from approximately 3×10^6 A549 cells were extracted in hypotonic cell lysis buffer (25 mm HEPES, pH 7.5, 5 mm $MgCl_2$, 5 mm EDTA, 5 mm dithiothreitol, 0.05% phenylmethylsulfonyl fluoride). Twenty microgram of proteins were added to caspase assay buffer (312.5 mM HEPES, pH 7.5, 31.25% sucrose, 0.3125% CHAPS) which uses benzyloxycarbonyl-DEVD-7-amido-4-(trifluoromethyl) coumarin as substrates (Calbiochem, Darmstadt, Germany). Release of 7-amido-4-(trifluoromethyl)coumarin (AFC) was quantified, after 2 h of incubation at 37 °C, using a Fluoroskan system (Thermo-Labsystems, Helsinki, Finland) set to an excitation value of 400 nm and emission value of 525 nm.

2.6. Analysis of cell death by propidium iodide (PI) fluorescence-activated cell sorting (FACS)

At indicated treatment(s), A549 cells were harvested through trypsinization, and washed twice with cold PBS (pH 7.4). The cells were centrifuged at 3000 rpm for 5 min, then the supernatant was discarded and the pellet was resuspended in 1× binding buffer at a density of 1.0×10^5–1.0×10^6 cells per mL. The sample solution (100 μl) was transferred to a 5 mL culture tube, and incubated with 5 μL of PI (Pharmingen) for 15 min at room temperature in the dark. Four hundred microliter of 1× binding buffer was added to each sample tube, and the samples were analyzed by FACS (Becton Dickinson) using Cell Quest Research Software (Becton Dickinson). Percentage of cell death was calculated as the number of PI positive cells divided by the total number of cells.

2.7. Generation of AMPKα knockdown stable A549 cells by lentiviral transfection

A549 cells were seeded to a six-well plate with 50–60% of confluence, 20 μl/ml of AMPKα shRNA containing lentiviral particles (a gift from Dr. Qingyou Zheng [19]) was added to each well, cells were cultured for 24 h. Cell culture medium was replaced with fresh puromycin (5.0 μg/ml)-containing medium every 2–3 days to select resistant colonies. The expression level of AMPKα was detected by Western-blots in the resistant colonies.

2.8. Adenoviral-mediated CA-AMPKα1 construct and transfection

The adenoviral vector expressing a constitutively active mutant of AMPKα1 (T172D, Ad-CA-AMPKα1) was a gift from Dr. Qingyou Zheng [19]. A replication-defective adenoviral vector expressing green fluorescence protein (Ad-GFP) was used as a control. Preliminary studies revealed that within 48 h of transfection with control Ad-GFP, 40–60% of A549 cells expressed GFP. Vectors (0.5 μg/ml) were transfected by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

3. Results

3.1. Chrysin activates AMPK in cultured lung cancer A549 cells

The primary aim of this study is to test the involvement of AMPK in chrysin's effect against lung cancer cells. To confirm AMPK activation after chrysin treatments in A549 cells, we used Western-blots to detect phosphorylation of AMPKα (Thr 172) and its downstream molecular acetyl-CoA Carboxylase (ACC, Ser 79). Results in Fig. 1A and B demonstrated a significant AMPK activation (AMPKα/ACC phosphorylation) in chrysin-treated A549 cells.

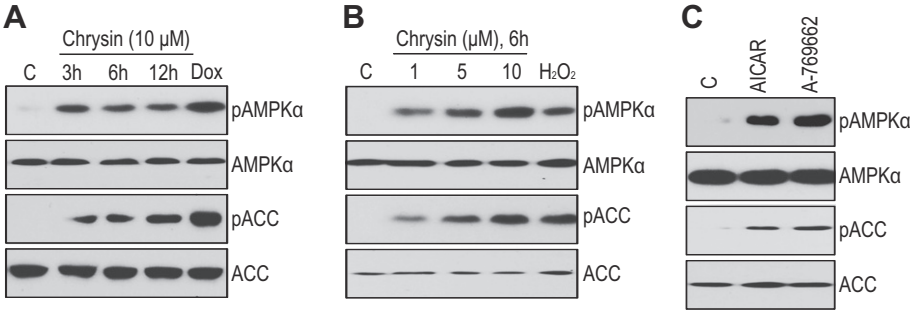

Fig. 1. Chrysin activates AMPK in cultured lung cancer A549 cells A549 lung cancer cells were either left untreated ("C") or incubated with indicated concentrations of chrysin for indicated hours (A and B), or with doxorubicin (1 μM, 2 h) (A), H_2O_2 (1 mM, 2 h) (B), AICAR (1 mM, 2 h) (C) or A-769662 (0.5 mM, 2 h) (C), cell lysates were then collected; p-AMPKα (Thr 172), AMPKα, p-ACC (Ser 79) and ACC were detected by Western-blots. Experiments in this figure were repeated three times to insure consistency of results.

Western-blot results showed a clear AMPK/ACC phosphorylation 3 h after chrysin incubation and it was last at least for 12 h (Fig. 1A). Further, in consistent with previous studies, we found doxorubicin (0.5 μg/ml, 2 h) (Fig. 1A) [18,20], H_2O_2 (1 mM, 2 h) [21] (Fig. 1B) and AMPK activators AICAR (1 mM, 1 h) (Fig. 1C) and A-769662 (0.5 mM, 1 h) (Fig. 1C) all activated AMPK in A549 cells.

3.2. Inhibition of AMPK diminishes chrysin-induced Akt/mTOR suppression, growth inhibition and apoptosis in A549 cells

Two different methods were used to inhibit AMPK in chrysin-treated A549 cells: shRNA-mediated gene silencing and adding the pharmacological inhibitors. AMPKα shRNA containing lentiviral particles were applied to knock-down AMPKα in A549 cells. Western-blots results in Fig. 2A showed that AMPKα was significantly knocked-down by target shRNA (12.1 ± 4.2% of control level) (Fig. 2A). Knocking-down of AMPKα inhibited chrysin-induced AMPK activation (AMPKα/ACC phosphorylation) (Fig. 2A), and cell death, apoptosis and growth inhibition were also inhibited (Fig. 2B–E). A549 cell growth was detected by the "MTT" cell viability assay (Fig. 2B) and the "clonogenicity" assay (Fig. 2C), cell death and apoptosis were reflected by changes of percentage of PI stained cells (Fig. 2D) and caspase-3 activity (Fig. 2E) respectively. In scramble shRNA-transfected control A549 cells, after 72 h of chrysin (10 μM) incubation, the percentage of viable cells decreased to 25.0 ± 4.1%, while in AMPKα shRNA-transfected cells, the number rose to 52.7 ± 2.6% (Fig. 2B). Meanwhile, the percentage of PI positive cells decreased from 36.9 ± 4.8% in control cells to 17.7 ± 2.8% in AMPKα-shRNA transfected cells (Fig. 2D). Further,

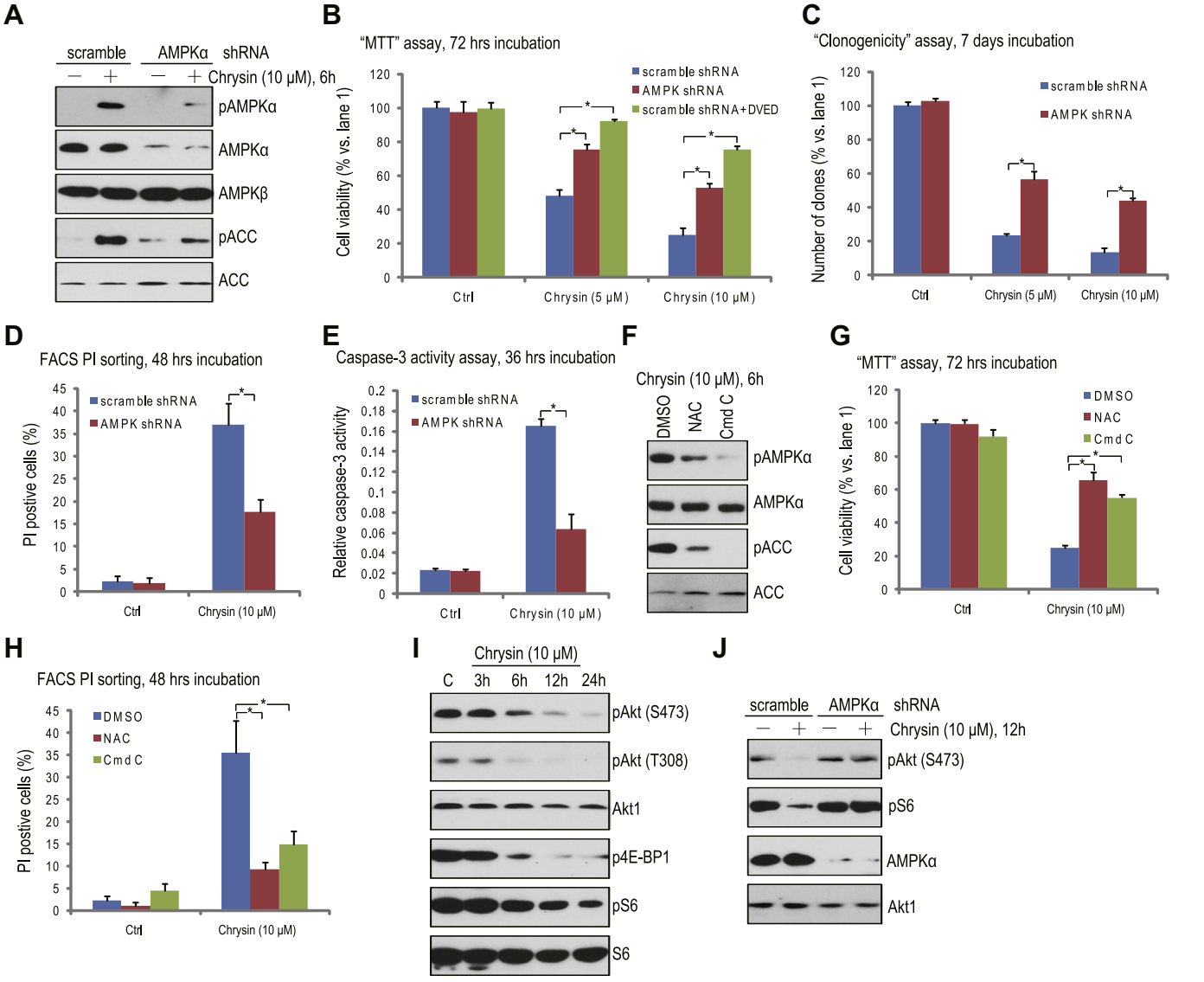

Fig. 2. Inhibition of AMPK diminishes chrysin-induced Akt/mTOR suppression, growth inhibition and apoptosis in A549 cells. Stable scramble or AMPKα-shRNA transfected A549 cells were exposed to chrysin (10 μM) for 6 h, p-AMPKα (Thr 172), AMPKα, AMPKβ, p-ACC (Ser 79) and ACC were detected by Western-blots (A). "MTT" cell viability assay (B) and "clonogenicity" assay (C) were used to test cell growth after indicated chrysin treatments in stable A549 cells expressing AMPKα or "scramble" (control) shRNA. Effects of caspase-3 inhibitor Z-DEVD-fmk (60 μM) on chrysin-induced A549 cell viability loss were also shown in (B), cell death was analyzed by FACS sorting PI positive cells (D) and caspase-3 activity assay (E). A549 cells were pre-treated with anti-oxidant NAC (0.5 mM), AMPK inhibitor compound c (Cmd C) for 2 h, followed by the indicated chrysin incubation, cell lysates were then collected and Western-blots was used to test AMPK activation (F),cell viability and death were analyzed by "MTT" assay (G) and FACS PI sorting (H). A549 cells were either left untreated ("C") or incubated with chrysin (10 μM) for indicated hours, activation of Akt/mTOR were detecting by Western blots using indicated antibodies (I). Stable scramble or AMPKα-shRNA transfected A549 cells were exposed to chrysin (10 μM) for 12 h, pAkt (Ser 473), pS6 (Ser 235/236), AMPKα and Akt1 were detected by Western-blots. Experiments in this figure were repeated three times to insure consistency of results. *$p < 0.01$ (ANOVA).

we found that both AMPK inhibitor compound C and anti-oxidant N-acetyl cysteine (NAC) inhibited chrysin-induced AMPK activation (Fig. 2F). Both agents also inhibited chrysin-induced cell viability loss (Fig. 2G) and cell death (Fig. 2H). Caspase-3 inhibitor z-DVED-fmk almost reversed chrysin-induced A549 cell viability loss (Fig. 2B), indicating that caspase-3 activation and apoptosis might be responsible for growth inhibition by chrysin in A549 cells. In line with previous findings [8], results in Fig. 2I demonstrated that chrysin inhibited the activation of Akt/mTOR (S6 and 4E-BP1 phosphorylation) in A549 cells, and knocking-down of AMPKα by shRNA almost restored Akt/mTOR activation (Fig. 2J). These results suggest that chrysin activates AMPK to inhibit Akt/mTOR activation, which might be responsible for growth inhibition and/or apoptosis.

3.3. Forced activation of AMPK mimics chrysin's in vitro anti-lung cancer cells effect

On the other hand, forced AMPK activation by introducing a constitutively active form of AMPKα (T172D, CA-AMPK) (Fig. 3A) inhibited Akt/mTOR activation (Fig. 3A). Further, CA-AMPK also inhibited normal A549 cell growth (Fig. 3B and C). "Clonogenicity" experiments in Fig. 3C showed the number of growth clones in CA-AMPK cells decreased to 25.8 ± 3.6% of the control vector transfected cells (Fig. 3C). Further, both AMPK activators 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and A-76922 inhibited A549 cell growth (Fig. 3D) and Akt/mTOR activation (Fig. 3F), while promoting cell death (Fig. 3E), and knocking-down of AMPK almost reversed these effects (Fig. 3D and E).

3.4. Chrysin increases doxorubicin-induced AMPK activation to promote A549 cell death and growth inhibition

A recent study showed that activation of AMPK by doxorubicin contributed to cytotoxicity and apoptosis in myocardial H9c2 cells [18]. Meanwhile, Ji et al., demonstrated that doxorubicin activates AMPK to promote cancer cell apoptosis, same study found that short-chain ceramides (C6) facilitated doxorubicin-induced AMPK activation to enhance cancer cell apoptosis and cytotoxicity [20]. Here, we confirmed the AMPK activation by doxorubicin in A549

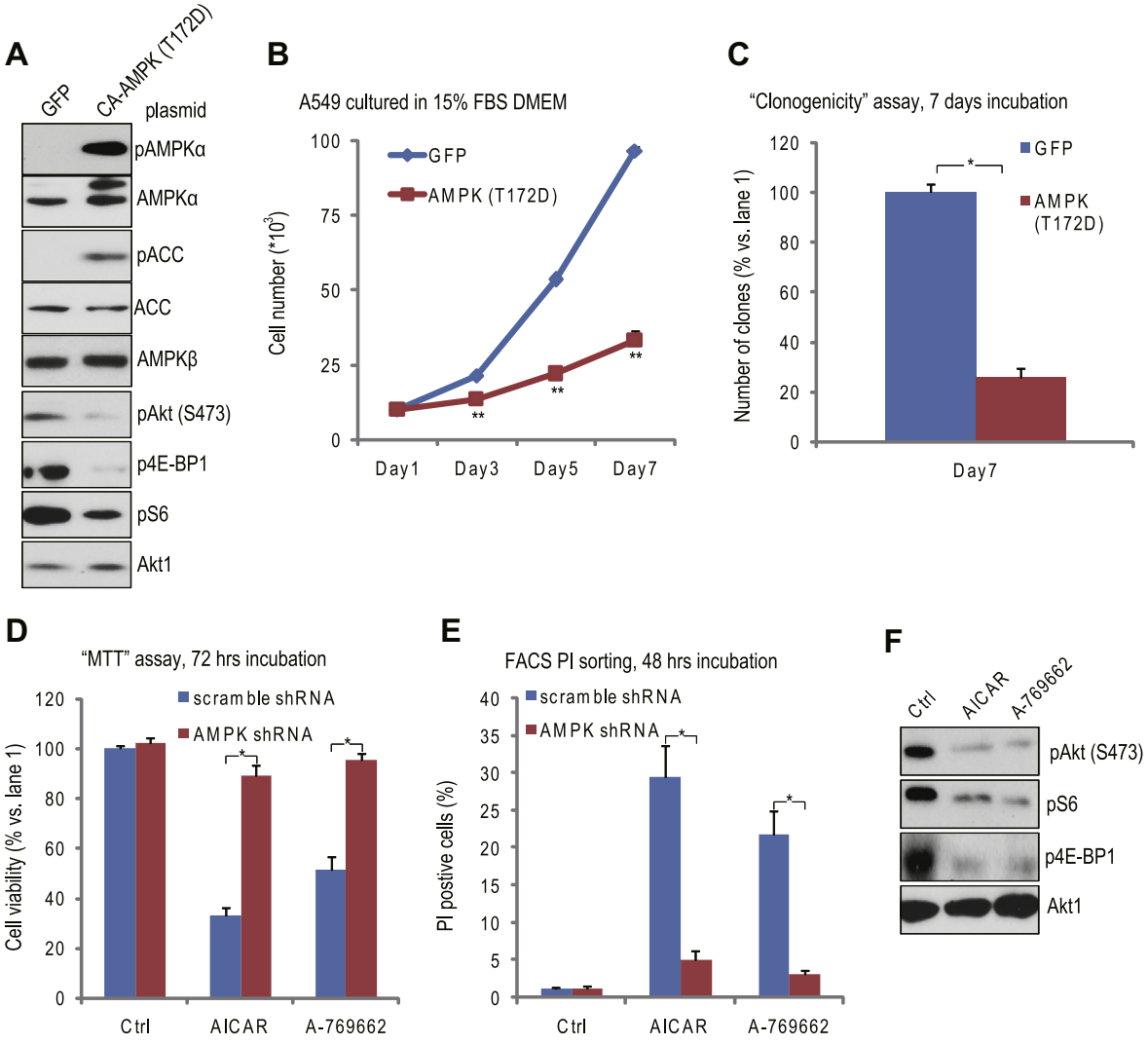

Fig. 3. Forced activation of AMPK mimics chrysin's in vitro anti-lung cancer cells effects. (A) Western-blots results confirmed successfully introducing CA-AMPK (T172D) to A549 cells, activation of Akt and mTOR in CA-AMPK transfected cells were also detected by Western-blots (A). 2×10^4 of CA-AMPKα (T172D) or vector control transfected A549 cells were cultured in DMEM with 15% FBS, for a total of 6 days, the total cell number was counted (B). Both types of cells also underwent a "clonogenicity" assay, and number of clones after 7 days of culture was recorded (C). Stable scramble-shRNA or AMPKα-shRNA transfected A549 cells were either left untreated or exposed to AICAR (1 mM), A-769662 (0.5 mM), cell viability was analyzed by "MTT" assay after 72 h (D), and cell death percentage was analyzed by FACS sorting PI stained cells after 48 h (E). A549 cells were either left untreated (Ctrl) or incubated with AICAR (1 mM,12 h) or A-769662 (0.5 mM, 12 h), pAkt (Ser 473), pS6 (Ser 235/236), p4E-BP1 (Ser 65), and Akt1 were detected by Western-blots (F). Experiments in this figure were repeated three times to insure consistency of results. $^*p < 0.01$ (ANOVA).

cells (Fig. 1A, Fig. 4C). A low dose of chrysin (1 μM) significantly enhanced doxorubicin-induced AMPK activation (Fig. 4C). Doxorubicin-induced A549 cell viability loss (Fig. 4A) and cell death (Fig. 4B) were also enhanced by chrysin co-administration. "MTT" results in Fig. 4A showed a 17.4 ± 1.8% loss of A549 cell viability after 3 days of chrysin (1 μM) treatment, and a 34.7 ± 1.3% loss by doxorubicin (1 μM) treatment, combination of the two caused a synergistic 69.7 ± 7.1% loss (combination index < 1). Percentage of PI positive cells increased to 27.8 ± 3.8% after the co-administration, compared to 6.4 ± 3.3% of the chrysin only treatment and 11.3 ± 5.3% of the doxorubicin only treatment (Fig. 4B). Knocking-down of AMPK by shRNA significantly reduced chrysin plus doxorubicin-induced growth inhibition in A549 cells, which suggests that activation of AMPK contributed to this process (Fig. 4D and E).

4. Discussion

The relationship of AMPK activation and cancer cell apoptosis has been established by a number of groups (See review [22]). Studies have confirmed that traditional chemotherapies including vincristine [23,24], taxol [25,26] and doxorubicin [18,20], as well as natural anti-cancer agents including ursolic acid [27], Honokiol [28] widdrol [14], EGCG [29] and fisetin [30], all activate AMPK to cause cancer cell apoptosis and cytotoxicity. Here, we found that AMPK activation might also be involved in chrysin-induced in vitro anti-lung cancer cells effect.

We found that anti-oxidant NAC largely inhibited chrysin-induced AMPK activation, which suggests that reactive oxygen species (ROS) might be the key regulator for AMPK activation by chrysin. Recently, studies have confirmed that chrysin is a potent inducer of ROS and in A549 and other cancer cells [31,32], and ROS is known to activate AMPK [21,33], thus it is possible that chrysin causes ROS accumulation in A549 cells, which then activates AMPK to cause cell apoptosis. The detailed mechanisms, however, need further investigation.

Doxorubicin is an important drug in the treatment of lung cancer and other solid tumors, but bone marrow and cardio-toxic effects of doxorubicin restrict its use in clinical medicine. A number of adjuvants have been tested to increase its chemo-efficiency. A recent study found that cell permeable short-chain ceramides (C6) facilitated doxorubicin-induced AMPK activation to enhance cell apoptosis [20], the authors suggested that agents that can activate AMPK might be beneficial to improve doxorubicin's chemo-efficienty [20]. Here, we found that chrysin facilitated doxorubicin-induced AMPK activation to promote A549 cell apoptosis. However, how exactly chrysin increases the chemo-sensitivity of doxorubicin and the role of AMPK in the process grantee further investigation. Further, also our current study

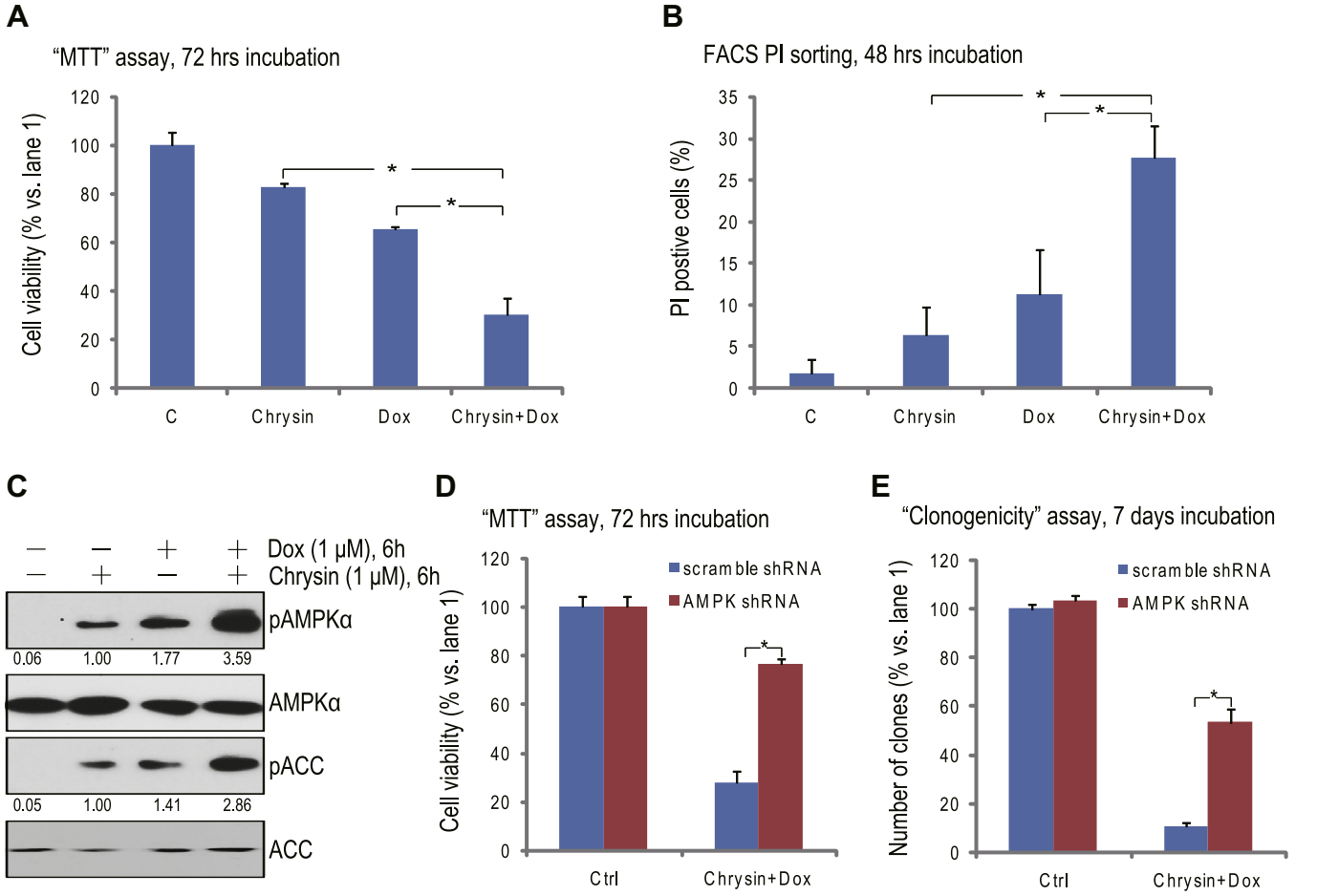

Fig. 4. Chrysin increases doxorubicin-induced AMPK activation to promote A549 cell death and growth inhibition. A549 cells were exposed to chrysin (1 μM), doxorubicin (Dox, 1 μM) or a combination of both, cell growth was analyzed by "MTT" assay (A), PI positive cells were sorted by FACS (B), activation of AMPK was detected by Western-blots (C). Stable scramble or AMPKα-shRNA transfected A549 cells were either left untreated or exposed to chrysin (1 μM) plus doxorubicin (1 μM) (Chrysin + Dox) for indicated time period, cell growth was analyzed by "MTT" cell viability assay (D) and "Clonogenicity" assay (E). Experiments in this figure were repeated three times to insure consistency of results. *$p < 0.01$ (ANOVA).

supports that AMPK activation by chrysin may cause Akt in-activation, future studies are needed to indentify other possible AMPK's downstream targets of cell apoptosis.

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