PNA-TPP Targeting Drp1 of Lung Adenocarcinoma Cancer Stem Cells Enhance the Sensitivity to Cisplatin

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PNA-TPP targeting Drp1 of lung adenocarcinoma cancer stem cells enhance the sensitivity to cisplatin

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Abstract

The presence of cancer stem cells (CSCs) is the source of occurrence, aggravation, and recurrence of lung cancer. Accordingly, targeting the lung CSCs has been suggested to be an effective approach for lung cancer treatment. Mitochondrial dynamics are currently considered to be closely related to apoptosis. Drp1 is the key component of the mitochondrial fission machinery. Our study indicated that peptide nucleic acid (PNA) promoting dynamic-related protein 1 (Drp1) expression improved cell apoptosis and the sensitivity to cisplatin (CDDP) in lung adenocarcinoma cancer stem cell. Interestingly, overexpression of Drp1 had a similar effect with PNA, and knockdown of Drp1 did the opposite. These data suggested that PNA promote cell apoptosis and enhance the sensitivity to CDDP of lung CSCs via Drp1. It will be of great interest to further explore the therapeutic strategies of lung cancer.

Keywords

PNA-TPP, Drp1, CSCs, Targeted therapy

Introduction

Lung cancer is the malignant tumor with the highest morbidity and mortality rate in the world. Only 18% of all patients with lung cancer are alive 5 years or more after diagnosis[1]. Adenocarcinoma is the most common histologic subtype of NSCLC for which chemotherapy is an common but important method of the treatment[1]. However, the drug resistance as developed during the course of therapy really limits the efficacy of chemotherapy. According to some studies, there exists a fraction of stem-like cells in tumor namely cancer stem cells (CSCs) which are the root of tumor growth, relapse, metastasis and drug resistance. In general, although conventional chemotherapeutic agents eliminate the majority of tumor cells, CSCs spared are capable of forming the heterogenic tumor mass[2,3]. Therefore, it is urgent to explore a effective treatment for targeting lung cancer stem cells.

Studies have shown that, mitochondrial fission has also been associated with a broad range of cell processes, such as apoptosis, mitochondrial biogenesis, mitophagy, cell proliferation, differentiation and transformation[4]. Ye et al. found that there was no significant change in the number of mitochondria in A549 stem cell spheres, in which mitochondrial fission decreased and fusion increased[5]. Mitochondrial fission is currently considered to be closely related to apoptosis and chemotherapy sensitivity in cancer cells[6-8]. Studies had shown that cisplatin resistance in cholangiocarcinoma was associated with mitochondrial dynamics, and mitochondrial fission induced apoptosis[9]. Mitochondrial fission is usually associated with decreased mitochondrial membrane potential, but it is usually recoverable. However, if the stress is too strong, the mitochondrial membrane potential cannot be restored and can be degraded by mitochondrial autophagy or eventually lead to apoptosis.

PNA, a DNA molecule analogue, in which sugar–phosphate backbone is instead by a pseudopeptide backbone composed of N-(2-aminoethyl)glycine units, can resistant to hydrolytic and enzymatic cleavage[10]. PNAs can specifically recognize complementary DNA obeying Watson–Crick base-pairing rules to form a stable helix[11]. Studies shown that specific peptide nucleic acid fragments targeting tumor-associated oncogene mutation genes, mitochondrial mutation genes, and epidermal growth factor receptor genes (EGFR) can inhibit growth of tumors such as melanoma, head, neck squamous cell carcinoma (HNSCC), non-small cell lung cancer (NSCLC), NCI-H446, breast cancer cell and human choriocarcinoma JAR Cells[12-15]. Mitochondrial DNA is a closed loop double-stranded DNA molecule, including the outer loop (heavy-strand promoter, HSP) and the inner loop (light-strand

promoter, LSP), which encodes oxidative phosphorylation related proteins including 12 species (ATP6 and ATP8, etc.) and 1 species (ND6), respectively[16]. Notably, both LSP and HSP are localized in D-loop regulatory region, making it a presumptive target for PNA interference. We thus designed a PNA for D-loop targeting, coupled with triphenylphosphonium (TPP), a delocalized lipophilic cation for mitochondria targeting[17,18]. Our groups had demonstrated that the given PNA-TPP significantly disrupted mitochondrial gene expression, resulting in cell death and enhancing chemosensitivity of cisplatin[19]. However, the specific mechanism of PNA-TPP promoting apoptosis of lung cancer stem cells and enhancing chemosensitivity of cisplatin is unknown.

In our study, firstly, resorting to the high mitochondrial membrane potential of the lung cancer cell line A549, we used PNA-TPP to block its mitochondrial DNA double-strand promoter consensus gene and disrupt the mitochondrial energy metabolism. Then we researched its influence on cAMP/PKA/Drp1 pathway, mitochondrial fission, ROS generation, chemosensitivity of cisplatin. The lentiviral transfection technique was used to alter the expression of Drp1 protein in the mitochondria of A549 cell spheres, and to investigate whether it affected the level of intracellular ROS and apoptosis. The research results are expected to provide new ideas and experimental basis for targeting the removal of lung adenocarcinoma stem cells and improving the clinical comprehensive therapeutic effect of lung adenocarcinoma.

Materials and methods

Reagents and antibodies

Cell culture products including Dulbecco's modified Eagle's medium (DMEM)/High Glucose,DMEM/F-12 and fetal bovine serum (FBS) were obtained from Biological Industries (Israel). Trypsin and EDTA were obtained from Solarbio (Beijing, China), B27 was purchased from Gibco (Grand Island, NY, USA), EGF and bFGF were obtained from Peprotech (Rocky Hill, USA). PKA-Cα and Drp1 Rabbit mAb antibody was obtained from Cell Signaling Technology (Danvers, MA); Bcl-2 and BAX Rabbit mAb antibody and β-actin mouse mAb antibody were purchased from proteintech (Wuhan, China). The cell counting kit-8 (CCK-8), goat anti-mouse/rabbit IgG (H&L) were purchased from Transgen Biotech (Beijing, China); the Annexin V-APC/PI apoptosis detection kit was obtained from keygen biotech (Nanjing, China);Western blot reagents were obtained from Bio-Rad Laboratories (Hercules, CA), and the Pierce BCA Protein Assay Kit was purchased from Applygen Technologies Inc (Beijing, China).

Cell line

The human NSCLC cell line A549 was obtained from the Second Affiliated Hospital of Nanchang university stored in liquid nitrogen preservation equipment and cultured in high glucose/DMEM supplemented with 10%FBS (v/v), streptomycin and penicillin , incubated with 5% CO2 at 37℃. A549 cell spheres were cultured using technique method as previously described. In a nutshell, A549 cells were seeded in the 6-well plates with 1000-1500 cells per well in 2ml 10%FBS/DMEM for 8-10 days. Thereafter, clones, namely meroclones, paraclones and holoclone, may formed, in which the latter possessing of some stem cell characteristics like continuous passage, and unlimited proliferation. So we next collected the holoclone cells and exchanged to culture in serum-free stem cell medium (SFM, contained F-12/DMEM, bFGF, EGF, insulin, and B27), 10-14 days after which primary A549 cell spheres were shaped. Finally we resuspended the primary spheres, and continued to grow in SFM to obtain secondary A549 cell spheres.

Synthesis and transfection of PNA-TPP complex

The PNA-TPP sequence designed and synthesized by PNAGENE company (daejeon, korea), was complementary to consensus sequence of human mtDNA L-chain and H-chain in D-loop region, which is 5'-CANACCGCCAAAAGA-3'. Then we transfected PNA-TPP diluted in SFM to a final concentration of 1nmol/mL in A549 cell spheres and incubated at 37°C, 5% CO2 without light exposure for 24 hours.

ATP content determination

ATP content determination was performed following the specification. Briefly, Equal number of cultured cells ($>10^6$)was collected for subsequent ATP analyses. The cells were resuspended in hot double-distilled water and cell membranes ruptured in boiling water bath for 10min, then mixed via vortex blending for 1 min to form ATP determination sample. The sample was collected for analyzing ATP by using a ATP Assay Kit (colorimetric). The absorbance was measured at 636 nm with Automatic microplate reader. ATP content was calculated by the following formula: ATP content (μmol/gprot)= (OD value of absorbance of treated group-OD value of absorbance of untreated)/(OD value of absorbance of standard group-OD value of absorbance of blank)*concentration of ATP standard sample (103μmol/L)*sample dilution multiple/sample protein concentration.

Western blot analysis

Total cellular proteins of A549 cells and A549 cell spheres under different treatments was isolated using RIPA lysis buffer with phenylmethanesulfony fluoride (PMSF) and mitochondrial proteins were extracted by a mitochondrial protein isolation Kit. The protein concentration was determined by the bicinchoninic acid (BCA) assay. Then proteins were diluted in SDS-PAGE protein loading buffer, a kind of 5× concentrated solution, to working concentration and denatured in boiling water. After that, identical quantity of proteins were electrophoretically separated by 8% or 10% sodiumdodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and the protein bands were transferred to polyvinylidene difluoride membrane (PVDF) membranes which blocked in 10% Skim Milk /TBST for 1-2 hours at room temperature. The PVDF membranes with protein bands were incubatd with appropriate primary antibodies dilute in 5%BSA/TBST at 4℃overnight. The membranes were incubated with PKA-Cα (1: 500), Drp1 (1: 500), Bcl-2 (1: 1000), Bax (1: 1000) and β-actin (1: 2000). The membranes were washed three times using 1xTBST 10 minutes once a time and incubated with corresponding HRP-conjugated secondary antibodies for 1-2 hours. After washed three times again, protein bands were detected with the ECL detection system and image lab software.

Determination of ROS production

Reactive oxygen species generation by astrocytes was detected with the fluorescent probe Dihydroethidium (DHE). Dihydtroethidium is a membrane-permeable compound, is oxidized to red fluorescent ethidium (DNA binding membrane-impermeable compound) by the action of radicals (O_2^-). The A549 cells and A549 cell spheres were incubated with 5 μM of DHE for 30 min at 37°C and protected from light. Finally, intracellular ROS production was determined by the increase in fluorescence observed by an fluorescence microscope under identical conditions in all experimental groups.

Design and interfection of lentiviral

Design of DRP1 short hairpin RNA (PL-DNM1L-SH1, PL-DNM1L-SH2, PL-DNM1L-SH3) from invitrogen cloned into lentiviral vector PL/IRES/GFP/U6, (PL-DNM1L-SH1, PL-DNM1L-SH2, PL-DNM1L-SH3) and synthesis of DRP1 (PL-DNM1L) was cloned into lentiviral vector pDS087-pL6-TO-V5-GIM. A549 cell spheres were cultured, subsequently Transfected with lentiviral vector. Cisplatin or PNA-TPP was added 72 hours later. After selection, cells were plated for proliferation and apoptosis.

Assessment of proliferation by CCK-8

Cell proliferation was detected with CCK-8 (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. In brief, A549 cell spheres were cultured in 96-well plates with three multiple holes under different treatment, and cell culture absorbance at 450 nm in each well was read by microplate reader after incubation for another 4 h.

Annexin V-APC/PI

After different treatments on A549 cell spheres, the percentage of apoptotic cells were determined with the Annexin V-FITC/PI staining following by the manufacturer's instructions. In addition, we used BD flow cytometer to analysis the cells positive after Annexin V-FITC/PI staining for 20min.

Statistical analysis

Experimental results of Western Blot were quantitatively analyzed by Image J software. β-actin was used to normalize the values of target protein. Every experiments was redid at least three times. Statistical analysis was performed by SPSS statistics for windows using two tailed Student's t test for comparison of two groups and data were expressed as mean ± standard deviation (mean± SD). In all experiments, if there was a $P<0.05$, differences were deemed statistically significant.

Results

Generation of A549 tumor spheres from A549

A549 cells formed three morphologically different colonies: holoclone, meroclone and paraclone by using single-cell cloning culture. Then, the holoclones were digested only and directly incubation with SFM. Three or four weeks later, A549 cells gradually formed into highly clustered tumor cell spheres, ie, A549 cell spheres (Fig 1). Our experiment shown that A549 cell spheres was higher expression level of PKA-Cα and Bcl-2 protein but lower mitochondria Drp1 and Bax than monolayer adherent growth A549 cells (Fig 2).

The PNA-TPP reduced the intracellular ATP levels and increased the intracellular ROS level in A549 cell spheres

Previous studies in our group showed that the levels of ROS and ATP in A549 cell spheres were lower than those in A549 cells. Most of the intracellular ROS and ATP are dependent on mitochondrial oxidative phosphorylation. Therefore, we designed a peptide nucleic acid-triphenylphosphine complexes (PNA-TPP) targeted to interfere with the A549 cell spheres mitochondrial light and heavy chain consensus sequence 5'-CANACCGCCAAAAGA-3'. After obtaining the corresponding OD value with microplate reader, the intracellular ATP content of A549 cell spheres in the blank control group

and PNA-TPP transfection group was calculated to be (125.20±10.90) μmol/gprot and (64.06±7.86) μmol/gprot, respectively. That is, PNA-TPP treatment reduced the intracellular ATP levels in A549 cell spheres as shown in Fig. 3. we determined the effect of PNA-TPP on intracellular ROS levels in A549 cell spheres using DHE-ROS fluorescence. The IOD values were obtained using Image Pro Plus 6.0 software analysis results.

PNA-TPP promoted Drp1 recruitment to mitochondrial to enhance cisplatin-induced apoptosis in A549 cell spheres via the cAMP/PKA pathway

Many studies have shown that mitochondrial fission is closely related to apoptosis. Studies have shown that disturbing of mitochondrial oxidative phosphorylation can promote apoptosis by affecting cAMP/PKA pathway and mitochondrial fragmentation. Phosphorylation at Drp1 S637 leads to an elongated mitochondrial morphology. Down-regulation of cAMP/PKA pathway can inhibit the phosphorylation of the 637 serine of Drp1, promote the transfer of Drp1 to the mitochondria, and promote mitochondrial fission. To verify this hypothesis, A549 sphere was treated with PNA-TPP for 24 hours, and then, the expression of mitochondrial Drp1 was detected. The data showed that the expression of PKA-Cα protein in A549 cell spheres was decreased and the expression of mitochondrial Drp1 protein was significantly increased upon PNA-TPP treatment, as compared with control group (Fig. 4 A-D).Additional, we found that PNA-TPP could promote the apoptosis level of A549 cell spheres and enhance the sensitivity to cisplatin. There was no significant change in the expression of bcl-2 and Bax protein between the blank control group and PNA-TPP group. However, compared with PNA-TPP or CDDP treatment alone, the expression level of bcl-2 protein in PNA-TPP combined with cisplatin group was significantly reduced, and the expression level of Bax protein was significantly higher(Fig. 4E-G). CCK8 showed that cell proliferation rate of A549 cell spheres were suppression after PNA-TPP treatment and the PNA-TPP combined with CDDP treatment group was less than that of PNA or CDDP alone treatment group (Fig. 5A, control, 100.0±0; PNA-TPP, 76. 85±4.72; CDDP, 74.15±5.99; PNA-TPP+CDDP, 51.80±5.13). Cell apoptosis rate of A549 cell spheres by flow cytometry after PNA-TPP treatment was increased and PNA-TPP combined with CDDP treatment group was higher than that of PNA or CDDP alone treatment group (Fig. 5B) control, 6.92±2.74; PNA-TPP, 14.44±2.76; CDDP, 16. 61±2. 31; PNA-TPP+CDDP, 32. 10±5. 67).

The effect of interfering with expression of Drp1 using lentivirus transfection technology on ROS level, the cell proliferation and apoptosis in A549 cell spheres

Drp1 played critical roles in ROS production, cell **apoptosis and** sensitivity to cisplatin. Given the above results, we hypothesized PNA-TPP can elevate levels of ROS, induce cell apoptosis and increase cisplatin chemotherapy sensitivity via regulation of Drp1. To prove this hypothesis, A549 cells were transfected with pLv-Drp1 and sh-Drp1 and selected with blasticidin to establish a stably cell line. Stably transfected cells were cultured in SFM to evaluate the role of Drp1 in A549 cell spheres (Fig. 6A-B). Our study showed the expression of Drp1 was increased and suppressed in A549 pLv-Drp1 and sh-Drp1 cell spheres (Fig. 6C-D). Consistent with the effect of PNA-TPP, the ROS level were increased in pLv-Drp1 A549 cell spheres. Similar with PNA-TPP transfection, the cell apoptosis were significantly more accelerated and had a higher proliferative ability in PLv-Drp1 A549 cell spheres than control group, which revealed the opposite result in sh3-Drp1 A549 cell spheres (Fig. 6E-G).

Discussion

Currently, cancer stem cells have been widely considered to drive tumorigenesis and tumor growth, drug resistance, and lead to cancer treatment resistance and recurrence. Studies proved that CSCs resistance to Chemotherapy can be obtained by, expression of ATP-binding cassette drug pumps, increased expression of anti-apoptotic proteins, resistance to DNA damaging agents. Our study. our study suggested that A549 cell sphere had the stronger ability of drug resistance, with higher expression of Bcl-2 protein and lower expression level of Bax protein. It was consistent with the above studies[20].

The results of this study showed that PNA-TPP treatment of lung cancer A549 stem cell spheres, PKA-Cα protein expression decreased, mitochondrial Drp1 protein expression increased, the intracellular ATP content decreased. Oxidative phosphorylation of mitochondrial inner membranes produces a majority of intracellular ATP by aerobic metabolism. F1F0-ATP synthase is a key enzyme that regulates ATP production. Two important subunits, ATP6 and ATP8, are transcribed and expressed by mitochondrial DNA[16]. Interfering with the mitochondrial DNA promoter consensus reduces the gene expression and inhibits ATP production. Drp1, is the key component of the mitochondrial fission machinery. Drp1 protein is encoded by nuclear DNA, distributed in the cytoplasm and mitochondria. However Drp1 activity is dependent on its activation by different post-translational modifications, including O-GlucNAcylation, SUMOylation, phosphorylation, S-nitrosylation, and glycosylation, and on the translocation from cytosol to mitochondria[21]. Evidence shows inhibition of the respiratory chain can lead to increased mitochondrial fission[22,23]. Mitochondrial DNA damage promotes mitochondrial fission, decreased mitochondrial membrane potential, reduced ATP synthesis, and increased release of cytochrome c[23]. It has been reported that if silence the death associated protein 3 (DAP3), the synthesis of oxidative phosphorylated protein encoded by mitochondrial DNA and the phosphorylation of Ser 637 in Drp1 are reduced, after which the mitochondrial fission promoted, which can reversed by enhancing the activity of PKA[24]. PKA holoenzyme consists of two regulatory subunits (R subunits) and two catalytic subunits (C subunits), PKA-Cα is one of the catalytic subunits. There is no activity in the case of holoenzymes, but it can be hydrolyzed to produce activity under the action of cAMP. A large number of studies have found that PKA-Cα is highly expressed in the serum of colorectal, lung, prostate, renal and adrenal glands, and lymphoma patients[25]. At the same time, it was found that human epidermal growth factor receptor-2 (HER2) is positive, especially for trastuzumab-resistant breast cancer patients with higher PKA-Cα protein expression levels[26]. The above shows that Drp1 and PKA are closely related to energy metabolism and may play a key role in tumorigenesis and drug resistance. Interestingly, activation of the cAMP/PKA signaling pathway activates target genes that play an important role in regulating stem cell self-renewal and differentiation[27,28]. The experimental results also showed that the level of PKA-Cα in A549 cell spheres was higher than that in A549 cells, and the expression of Drp1 protein in mitochondria was reduced. This is consistent with the above findings. At the same time, the SANC study of cardiac sinus node cells found that cAMP synthesis requires the consumption of ATP, and interference with mitochondrial oxidative phosphorylation can reduce cAMP production by inhibiting ATP[29]. In summary, we believe that the possible mechanism of PNA-TPP inhibiting mitochondrial fission in A549 cell spheres is that PNA-TPP targets mitochondrial oxidative phosphorylation in the mitochondria, reduces ATP production, inhibits cAMP/PKA pathway activation, and mediates Drp1 position 637 Serine phosphorylation is reduced and Drp1 protein is localized in mitochondria, eventually leading to increased mitochondrial fission.

The results of this experiment show that when PNA-TPP is targeted to enter the mitochondria, mitochondrial Drp1 protein increases, ROS production also increased, the rate of apoptosis increased,

the cell proliferation capacity weakened. Mitochondrial splitting and apoptosis contribute to mitochondrial fission induced by excessive ROS-induced oxidative stress. Lipopolysaccharide (LPS) stimulated the increase of Drp1 protein expression and promoted the mitochondrial fission and ROS production of microglia. The expression of mitochondrial fission inhibitor 1 (Mdivi-1) and silencing Drp1 can reverse the above-mentioned effects of LPS, and then stimulate downstream NF-κB and MAPK pathways to exert biological effects[30]. Our study also found that overexpression of Drp1 promoted ROS production, decreased cell viability and increased apoptosis, which was similar to PNA-TPP, and down-regulation of Drp1 did the opposite. However, PNA-TPP can also promote ROS production by interfering with mitochondrial energy metabolism. Interfere with chronic lymphoid leukemia cells F1F0-ATPase, promote ROS production and apoptosis[31]. In conclusion, PNA-TPP may partially inhibit mitochondrial fission, promote ROS production, resulting in oxidative stress and promoting apoptosis, nuclear transcription factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathway may play a role. However, the specific mechanism needs further study.

Currently platinum-based chemotherapy is still the first line of treatment for most lung cancer patients. However, the problem of drug resistance has become increasingly prominent. At present, most studies have confirmed that reversal of drug resistance in tumors depends on mitochondrial fission and increased ROS production. There is a colocalization of Drp1-Bax in the mitochondrial cleavage site, mitochondrial division can decrease Bcl-2 expression and up-regulate Bax, and small interfering RNA (siRNA) inhibits Bax transfer to mitochondria[32]. However, our results showed that PNA-TPP inhibited mitochondrial fission of lung cancer stem cells, but Bcl-2 protein expression and Bax expression did not change significantly. This may be due to the fact that mitochondrial fission in lung cancer stem cells may affect Bax and Bcl-2 proteins through other proteins other than Drp1, or that Drp1 may promote apoptosis by regulating other apoptosis mechanisms, but its apoptosis mechanism is to be further studied. Studies have shown that by inducing elevated ROS production in rectal cancer cells, down-regulating Bcl-2 and up-regulating Bax protein expression can promote apoptosis, reverse multidrug resistance of rectal cancer, and enhance chemosensitivity[33]. This is similar to our experimental results. Our research results indicated compared with PNA-TPP or cisplatin alone, the PNA-TPP combined with cisplatin treatment group expressed higher levels of Bax and lower levels of Bcl-2, and increased apoptosis rate and decreased cell proliferation ability. This suggests that PNA-TPP may enhance cisplatin chemosensitivity by promoting mitochondrial fission and increasing ROS levels. Cisplatin can induce oxidative stress by inducing ROS production during chemotherapy, leading to DNA damage in tumor cells and triggering a cascade of apoptosis-related cascades. Due to cancer stem cells have stronger DNA repair ability, which may be one of the reasons why cisplatin chemotherapy is not sensitive. In this experiment, PNA-TPP was able to induce oxidative stress and cause excessive DNA damage, so that the lung adenocarcinoma A549 stem cell sphere could not be repaired in time.

Conclusion

In summary, PNA-TPP may partially regulate the cAMP/PKA/Drp1 pathway, promote mitochondrial division and ROS production, promote apoptosis of lung cancer stem cells, and enhance chemotherapeutic sensitivity of cisplatin. This may provide a theoretical basis for lung adenocarcinoma stem cell targeted therapy.

Ethics approval and consent to participate

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

Consent for publication

Informed consent For this type of study, formal consent is not required.

Availability of data and materials

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

Competing interests

All authors declare no conflict of interest

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Authors' contributions

Lixia Xie and Xiaoqun Ye developed and designed the study and analyzed the data and wrote the initial draft of the paper Lixia Xie, Ping Hu, Yong Cheng, Xiaoqun Ye performed the experiments Yong Cheng and Ping Hu performed the statistical analyses All authors accepted the final version of this manuscript for publication

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Illustration

Fig 1 The morphology of A549 cells during different culture phase and (A) A549 cells grew adherently in 10%FBS/DMEM medium. (B) Holoclone derived from single parental cell of A549 cells under 10%FBS/DMEM medium. (C) Primary A549 cell spheres formed in the SFM/F-12 medium. (D) Secondary A549 cell spheres derived from single parental cells of primary A549 cell spheres formed in the SFM/F-12 medium. (A, B, C) and D 200× under microscopy.

Fig 2 The level of Drp1 protein in mitochondria and (PKA-Cα, Bcl-2, Bax) protein were detected on A549 cells and A549 cell spheres. (A) Bands showed the protein expression of Drp1 (upper panel), and COX IV (lower panel). (E) Bands from top to bottom showed the protein expression of PKA-Cα, Bcl-2, Bax and β-actin, respectively. (B) and (C,D,F), The quantification results of the western blot respectively with normalization relative to COX IV or β-actin expression. Data are taken from 3

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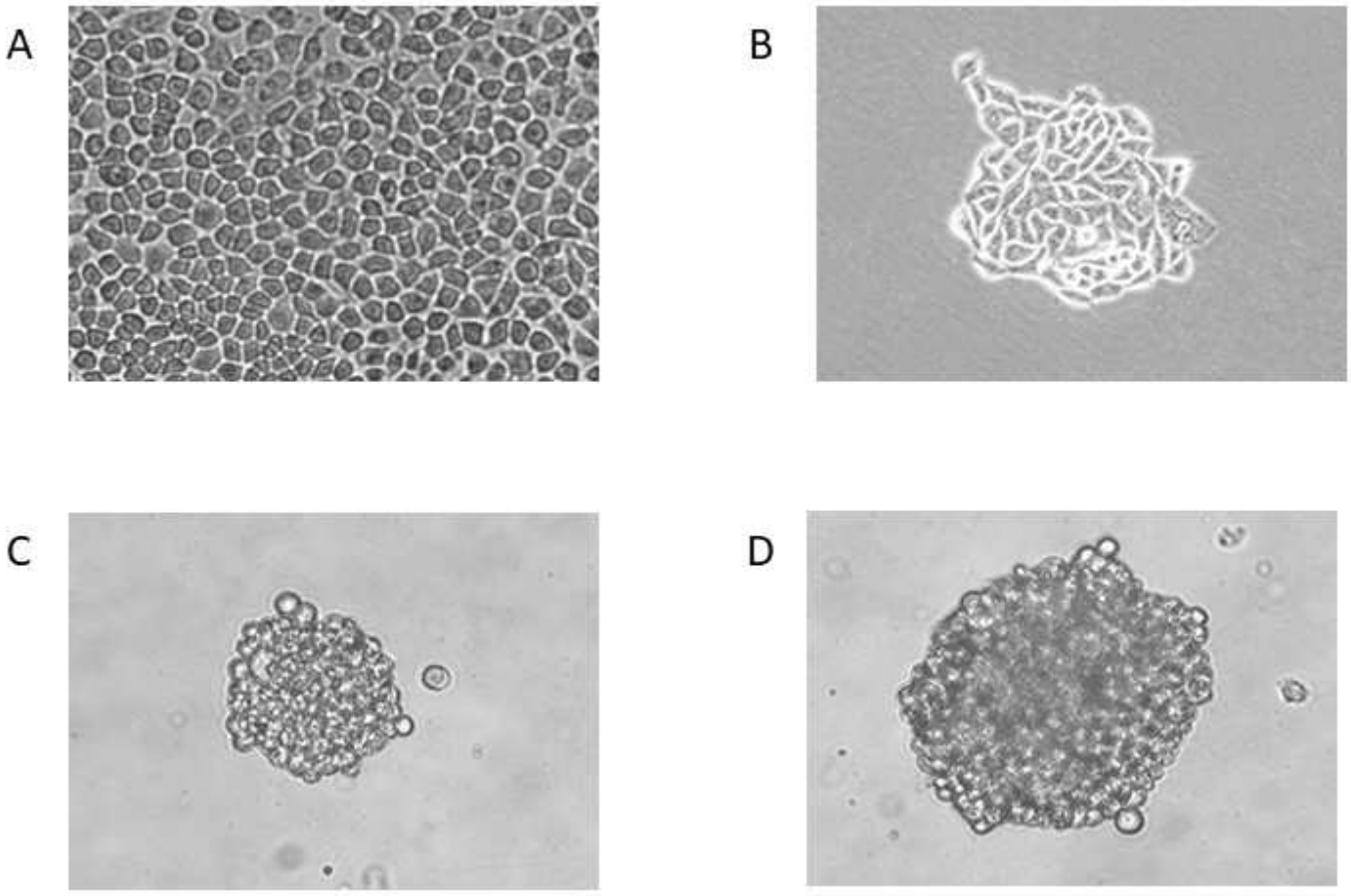
Fig 3 A and B show the ATP level was reduced and the ROS level was increased in A549 cell spheres with PNA-TPP treatment, respectively. A549 cell spheres were teratment with PNA-TPP for 24 hours, then ATP and ROS level was measured in cytosol by ATP and ROS detection kit.

Fig. 4 The level of Drp1 protein in mitochondria and and (PKA-Cα,Bcl-2, Bax) protein were detected on A549 cell spheres with different treatment. A,The protein expression of Drp1 in mitochondria treatment treatment with or without PNA-TPP in A549 cell spheres were detected by Western blot. Representative bands show the protein expression of Drp1 (upper panel) and COX IV (lower panel). B,The protein expression of PKA-Cα treatment with or without PNA-TPP of A549 cell spheres were detected by Western blot. Representative bands show the protein expression of PKA- Cα (upper panel) and β-actin (lower panel).E,Bcl-2 and Bax levels were determined by Western blotting. A549 spheres were incubated with o.5μg/ml CDDP or PNA-TPP for 24 h. Representative bands show the protein expression of Bcl-2 (first panel), Bax (second panel), and β-actin (lower panel). C,D,F,G,The histograms showing quantification of those bands, normalization relative to COX IV or β-actin expression. The data are resented as the mean± standard deviation. of three separate experiments. (ns) P＞0.05,*P<0.05,*P<0.01,***p<0.001, versus the control group.Mito, mitochondria.

Fig. 5 A and B show PNA-TPP and CDDP affecting the proliferation and apoptosis of A549 cell spheres. A, CCK-8 showed the cell proliferation inhibition rate while A549 cell spheres were transfected with PNA-TPP for 24 hours,after which 0.5 μg/ml CDDP for 24 hours. B, A549 cell spheres after different treatmentswere collected and stained by Annexin-V/PI, percentage of cell apoptosis was determined by flow cytometry. Quantification was performed on three independent experiments. Data are presented as mean ± standard deviation.Data were taken comparisons were carried out by Student's t-test. **P<0.01; ***P<0.01.PNA-TPP, CDDP and PNA-TPP+CDDP versus control, respectively.

Fig. 6 Overexpression or knockdown of Drp1 in A549 cell spheres affected ROS level, cell the proliferation and apoptosis. A and B show the transfection rate was over 80% in all experiments. C and D, Testing the efficiency of pLv-Drp1 and different ShRNAs to knockdown endogenous Drp1 in A549 cell spheres in transiently transfection experiments. E, The ROS level was increased and reduced in A549 cell spheres when overexpression and knockdown Drp1, respectively. F, overexpression and knockdown Drp1 may suppress and promote cell proliferation in A549 cell spheres, respectively.G, overexpression and knockdown Drp1 may promote and suppress apoptosis in A549 cell spheres, respectively. CT, control; D-, Drp1 negative control for overexpression using the pDS087-pL6-TO-V5-GIM vector; D+, Drp1 overexpression using pDS087-pL6-TO-V5-GIM vector; Sh-, negative control for knockdown expression of Drp1 using the PL/IRES/GFP/U6; Sh(1–3)+, Sh-RNAs targeting different regions of Drp1 transcript knockdown expression of Drp1 using the PL/IRES/GFP/U6. Quantification was performed on three independent experiments. Data are presented as mean ± standard deviation. Data were taken comparisons were carried out by Student's t-test. (ns) P＞0.05,*P<0.05,*P<0.01,***p<0.001 **P<0.01;D- and Sh- versus CT ,respectively,D+ versusD-,Sh- versus Sh(1-3)+.

Figures



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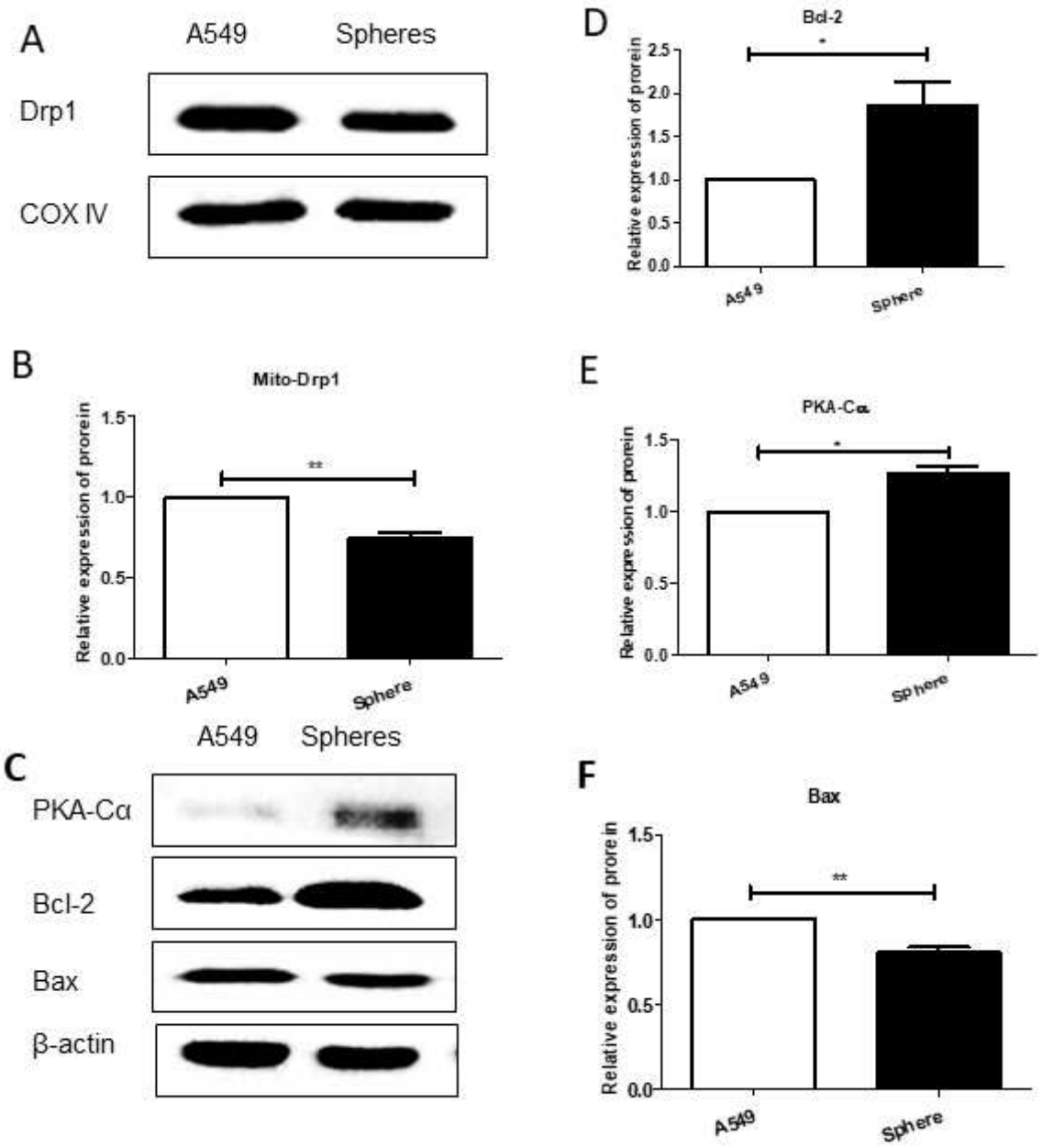

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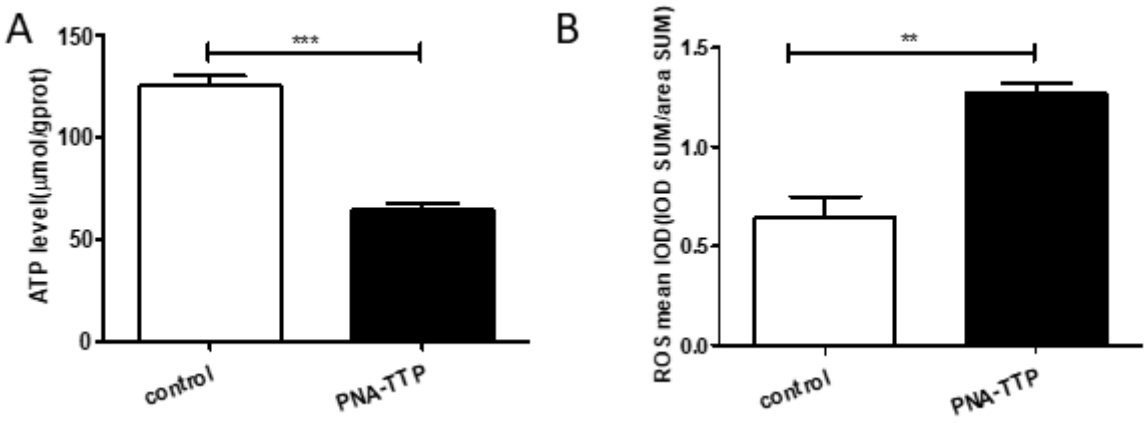

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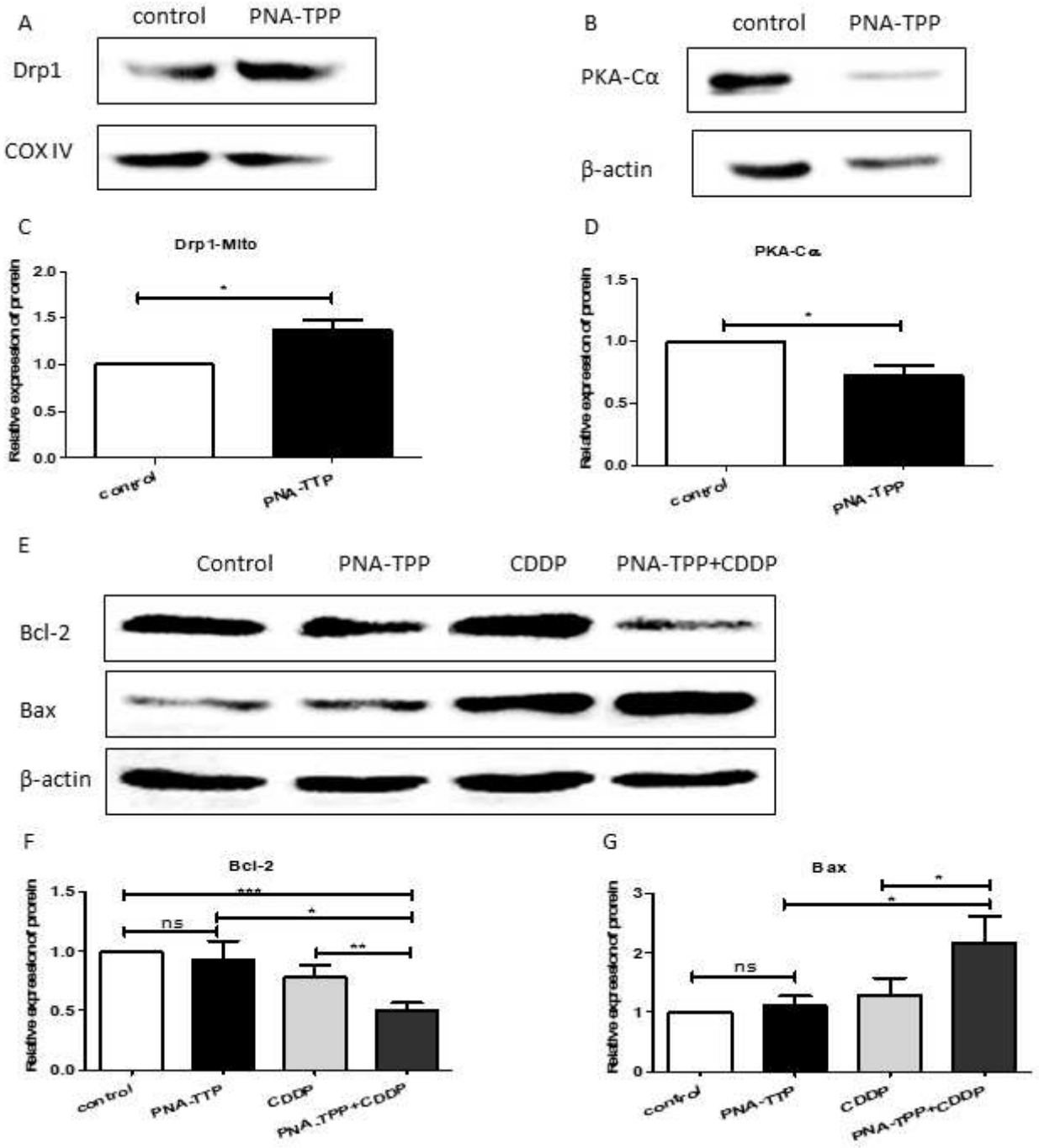

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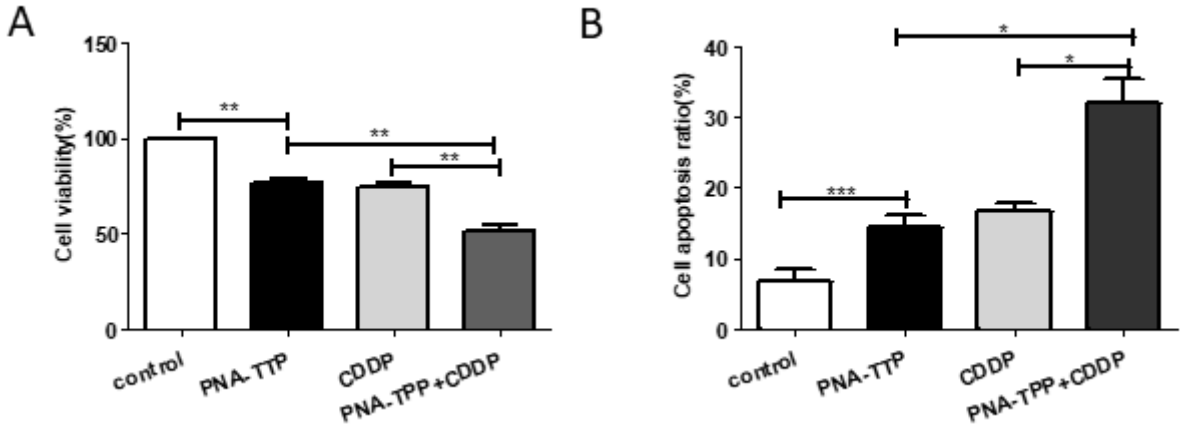

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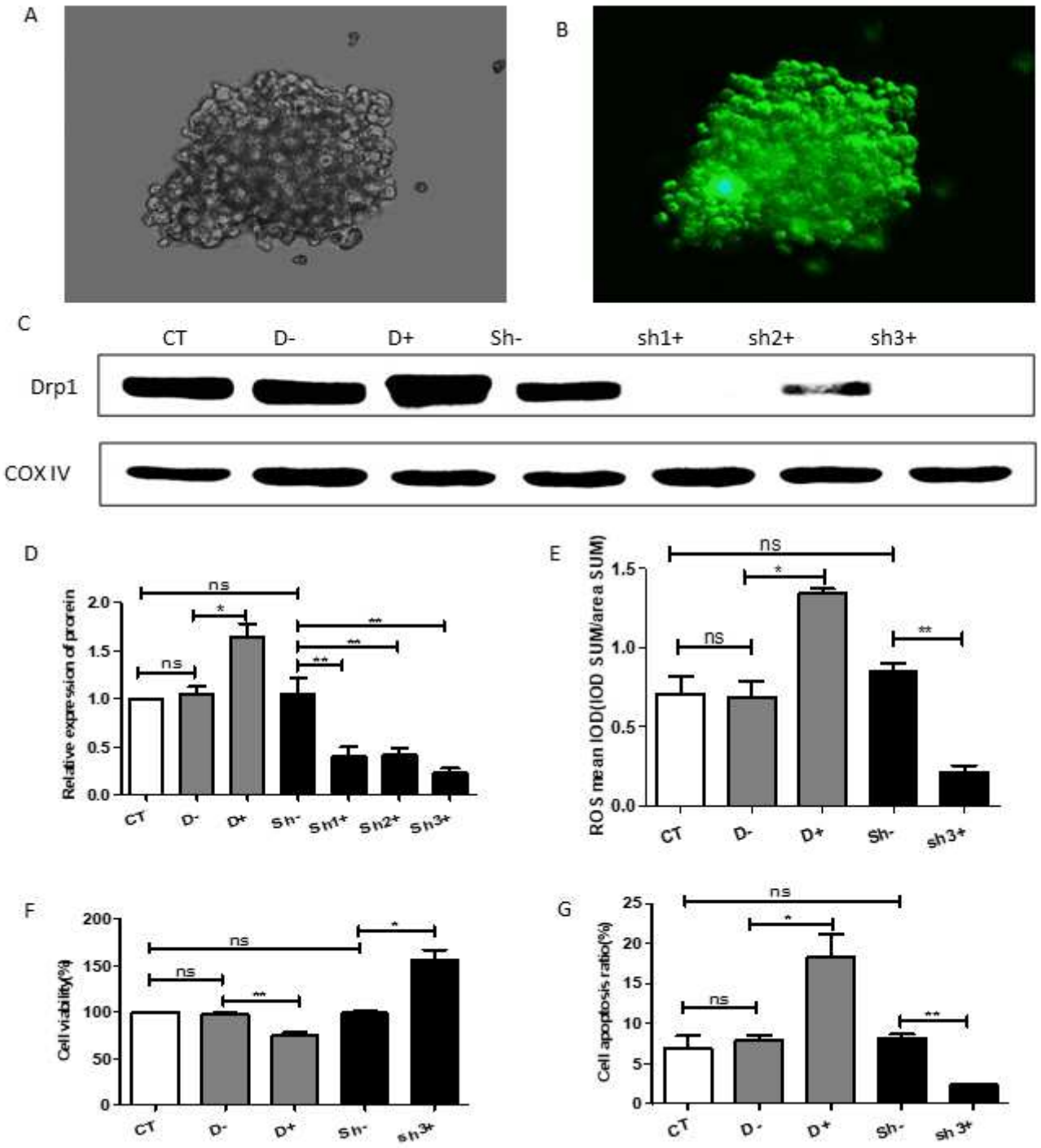

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