Supplemntary information

materials and Methods

Reagent

Luteolin (Sigma-Aldrich, CA, USA) was diluted to 10 μmol/L in the Dulbecco's modified Eagle's medium (DMEM), and this concentration was used in all assays detailed below.

Cell growth analysis

To evaluate the effect of luteolin exposure on cell growth, about 2×10^3 oral carcinoma 3 (OC3) cells were seeded in 96-well plates and allowed to adhere overnight. On the next day, cells were incubated in culturing medium alone, culturing medium containing dimethyl sulfoxide (DMSO) or 10 μmol/L of luteolin, respectively, and proliferation rates were monitored at 24, 48, and 72 h using a cell counting kit-8 (CCK8; Yeasen Biotechnology, Shanghai, China), according to the manufacturer's instructions. Briefly, 10 μL of CCK8 solution was added to each well at indicated time points, and after 1h incubation at 37 °C, absorbance value was measured at 460 nm using a microplate reader Synergy H1 (BioTek, VT, USA).

Transwell migration assay

To examine the effect of luteolin exposure on cellular migration capacity, about 2×10^4 OC3 cells were seeded in the upper chamber (4.5 μmol/L pore size; Corning Costar, NY, USA) containing culturing medium alone, culturing medium with DMSO or 10 μmol/L luteolin, respectively, and in the lower chamber, 800 μL of culturing medium supplied with 10% (volume fraction) FBS (Gibco, CA, USA) was added. After 24 h incubation at 37 °C, non-migratory cells remaining in the upper surface of the membrane filter were scraped off using a cotton swab, and then migrated cells were fixed in 4% paraformaldehyde (Sangon Biotech, Shanghai, China) followed by a crystal violet (i.e., 0.1% (volume fraction), Sangon Biotech) stain prior to manual counting under an inverted light microscope.

Cell cycle analysis

A flow cytometry-based assay was performed to investigate the effect of luteolin exposure on cell cycle progression. Specifically, about 1×10^6 OC3 cells were seeded in 6-well plates, and the cell cycle was firstly synchronized to the G1 stage using 10 μmol/Lol/L of lovastatin (Sigma-Aldrich). On the next day, cells were washed three times in phosphate-buffered saline (PBS) (Sangon Biotech) and then incubated in culturing medium alone, culturing medium with DMSO (Sigma-Aldrich) or 10 μmol/L luteolin, respectively. After 24 h incubation, cells were washed in PBS three times and then fixed in 70%

ice-cold ethanol at 4 °C. After overnight fixation, cells were washed twice in PBS and stained with propidium iodide (PI, BD Biosciences, CA, USA) at room temperature for 15 min before analyzing cell cycle progression on a flow cytometer (BD Biosciences, CA, USA).

Western blot analysis

To further investigate the change of expression of key molecules involved in cell cycle progression following luteolin exposure, about 2×10^6 OC3 cells were treated with 10 μmol/Lol/L of luteolin diluted in culturing medium for 24 and 48 h, respectively. As controls, cells incubated in culturing medium alone or culturing medium containing DMSO were also included. Cells were washed thrice in PBS and lysed in 50 μL of radioimmunoprecipitation assay buffer (Beyotime Biosciences, Shanghai, China). After centrifugation at 12 000 *g* for 15 min at 4 °C, supernatants were collected, and protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, MA, USA). Collected supernatants were mixed with an equal volume of reducing buffer (Bio-Rad, CA, USA) containing 5% β-mercaptoethanol (Bio-Rad). After 15 min boiling, about 10 μg of protein was loaded and resolved via 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked in 10% skim milk for 1 h at room temperature and then probed with anti-PLK1 (1:1000, volume ratio (the same below); Santa Cruz Biotechnology, CA, USA), anti-p53 (1:1000; Santa Cruz Biotechnology), anti-p53-Ser15(p) (1:1000; Santa Cruz Biotechnology) and anti-GAPDH (1:1000; Boshide Biotechnology, Wuhan, China), respectively. After overnight incubation at 4 °C, membranes were washed three times in Tris-Buffered Saline that contains 5% Tween®20 (TBST; Thermo Fisher Scientific) followed by 1 h incubation with HRP-conjugated goat-anti-mouse/rabbit secondary antibodies (1:1000; Bio-Rad) at room temperature. Immunoreactive bands were visualized using SuperSignal™ West Pico PLUS Chemiluminescent Substrate kits (Thermo Fisher Scientific) and imaged on the chemiluminescent immunodetection system (ChemiDoc XRS, Bio-Rad). All obtained images were analyzed using ImageJ software (National Institutes of Health, MD, USA) for calculating band intensities.

Measurement of bioenergetic in luteolin-exposed oral carcinoma 3 cells

To further dissect metabolic phenotypes of OC3 following luteolin exposure, changes in extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of OC3 cells were monitored in real-time using Seahorse XFe Extracellular Flux Analyzer (Agilent, CA, USA). Briefly, OC3 cells were seeded at a density of about 3×10^4 cells/well and treated with 10 μmol/L of luteolin diluted in culturing medium.

As controls, cells were also incubated in culturing medium alone or culturing medium containing DMSO. After 24 h treatment, the culturing medium was replaced with reagents provided by Seahorse XF Glycolytic Rate Assay Kit (Seahorse Bioscience, MA, USA) and Seahorse XF Cell Mito Stress Test Kit (Seahorse Bioscience) before the measurement of ECAR and OCR, respectively. Cells were allowed to stabilize for 18 min, during which basal levels of OCR and ECAR were measured at 0, 6, 12, and 18 min. For OCR measurement, oligomycin, carbonyl cyanide 4-[trifluoromethoxy] phenylhydrazone (FCCP), and rotenone, which are specific mitochondrial inhibitors and activators, were sequentially injected at 18, 42, and 60 min, respectively. For ECAR measurement, glucose, oligomycin, and 2-deoxyglucos (2-DG) were sequentially injected at 18, 36, and 54 min, respectively. OCR and ECAR were recorded every 6 min to the end of the experiment (i.e., 72 h). All data were analyzed using Seahorse Wave software (Aligent) and exported to Excel (Microsoft) for further analysis.

Measurement of adenosine triphosphate and reactive oxygen species

To further quantify the production of mitochondrial metabolites in OC3 cells following luteolin treatment, cellular adenosine triphosphate (ATP) and reactive oxygen species (ROS) levels were measured. Specifically, about 1×10^6 cells were incubated in culturing medium alone, culturing medium containing DMSO or 10 μmol/L luteolin, respectively. After 24-h incubation, cells were collected to assay ATP and ROS levels using the Enhanced ATP Assay Kit and Reactive Oxygen Species Assay Kit (Beyotime Biosciences), respectively, according to the manufacturer's instructions.

Statistical analysis

All data are presented as mean±standard deviation. Statistical differences between the two groups were evaluated using Student's *t*-tests. Statistical significance was defined as $P<0.05$.