Supplementary information

Fucoidan sulfate from *Sargassum fusiforme* regulates the SARS-CoV-2 receptor AXL expression in human embryonic lung diploid fibroblast cells

Xuqiang ZHOU[1], Weihua JIN[3], Di JIANG[3], Yipeng XU[4], Sanying WANG[2], Xinna WU[5], Yunchuang CHANG[6], Huili SU[2], Tianjun ZHU[2], Xiaogang XU[2], Genxiang MAO[1,2]

[1]*College of Life Science, Zhejiang Chinese Medical University, Hangzhou 310053, China*
[2]*Zhejiang Provincial Key Lab of Geriatrics & Geriatrics Institute of Zhejiang Province, Department of Geriatrics, Zhejiang Hospital, Hangzhou 310030, China*
[3]*College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, China*
[4]*Zhejiang Cancer Hospital, the Key Laboratory of Zhejiang Province for Aptamers and Theranostics, Institute of Basic Medicine and Cancer (IBMC), Chinese Academy of Sciences, Hangzhou 310022, China*
[5]*School of Medicine, Zhejiang University, Hangzhou 310058, China*
[6]*School of Biological and Chemical Engineering, Zhejiang University of Science and Technology, Hangzhou 310023, China*

Methods and materials

1 Cell culture

Human embryonic lung diploid fibroblast cells (2BS) were established and donated by the China Institute of Biological Products (Beijing, China) (Li et al., 1995; Zheng et al., 2006; Mao et al., 2012). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) basic medium containing 10% (volume fraction) fetal bovine serum (FBS) (Life Technologies Corporation, USA) and 1% (volume fraction) penicillin/streptomycin (Shanghai Biyuntian Biotechnology Co., Ltd., Shanghai, China). The cells were passaged with 0.25% (2.5 g/L) trypsin-ethylene diamine tetraacetic acid (EDTA) (Life Technologies Corporation) when there was no gap between them and incubated for 24 h before the experiment.

2 Preparation and purification of fucoidan sulfate

Extraction was performed using hot water according to a previously reported preparation method (Jin et al., 2020). *Sargassum fusiforme* (*S. fusiforme*) was extracted by acid extraction (0.1 mol/L hydrochloric acid) and alkali extraction (5% (0.05 g/mL) Na_2CO_3), and SFW, SFS, and SJF were obtained. The crude polysaccharides obtained by water extraction were purified further. The sample was then purified by anion exchange chromatography on a DEAE-Bio Gel Agarose FF gel eluted with water (5 L) (SFW-1), 0.5 mol/L NaCl (5 L) (SFW-2), and 2 mol/L NaCl (5 L) (SFW-3). The crude polysaccharide obtained by acid was separated through anion exchange chromatography on a DEAE-Bio Gel Agarose FF gel and eluted using water (SFW-4).

Dilute acid degradation: The eluted components of the 0.5 and 2 mol/L sodium chloride solution were degraded by dilute acid degradation. After that, the molecular weight of the sample was separated by Bio-Gel P-4 Gel, and the mobile phase was a 0.5 mol/L ammonium bicarbonate

solution. The components were concentrated, desalted, and freeze-dried to obtain SFW-2-M, SFW-2-S, SFW-2-O, SFW-3-M, SFW-3-S, and SFW-3-O.

Oxidative degradation: The eluted components of 0.5 mol/L and 2 mol/L sodium chloride solutions were oxidized and degraded by hydrogen peroxide to obtain low molecular weight components, concentrated, freeze-dried, and preserved to obtain SFW-2-D and SFW-3-D.

3 Senescence-associated β-galactosidase staining

Senescence-associated β-galactosidase staining was performed according to the GENMED cell senescence-associated β-galactosidase detection kit. The general steps were as follows: the GENMED staining solution was diluted 20 times with GENMED's diluent and preheated at 37 °C. The old medium was discarded, and 1 mL of GENMED cleaning solution was added to each well, washed once, and the cleaning solution was discarded. After that, 1 mL of GENMED's fixing solution was added to each well. The solution was discarded after incubation at room temperature for 5 min. Then, 1 mL of acid solution was added to each well. After cleaning the cell surface, the acidic solution was discarded. The acidic solution was discarded after repeated cleaning. Staining solution preheated to 1 mL was added to each well to cover the cell surface. The cell culture plate was placed in a carbon dioxide incubator and incubated for 3–16 h, depending on the state of the cells. The cell culture plate could be removed and observed every 3–4 h. The positive cells were observed under a microscope. Senescence-associated β-galactosidase presented blue under the microscope.

4 Detection of reactive oxygen species (ROS)

Human embryonic lung diploid fibroblast cells within population doubling 30 (PD30) and PD55, respectively. The cells were treated when they grew to 50% density in a six-well plate. The culture medium was then absorbed and washed three times with phosphate buffered saline (PBS). An active oxygen-free radical fluorescence probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA), with a molar concentration of 10 μmol/L in 1 mL, was added to each well and incubated in a cell incubator for 20 min. The probe was removed and washed three times with DMEM basic medium. Cells were then digested with trypsin and washed once with PBS. The cells were collected and resuspended in 500 μL PBS. Lastly, the cells were transferred to a flow tube for detection.

5 Cell viability test

The effect of fucoidan sulfate on the activity of 2BS cells was detected using a CCK-8 kit. Human embryonic lung diploid fibroblast cells in the logarithmic growth phase were inoculated into a 96-well plate at a density of 1×10^4 cells/100 μL per well, cultured overnight in a CO_2 incubator, mixed with fresh medium containing different concentrations of fucoidan sulfate, and the old culture medium was removed from the 96-well plate. The fucoidan sulfate samples were dissolved in sterile water and then filtered with a 0.22 μm filter membrane. Fresh medium containing fucoidan sulfate sample was added and cultured in a CO_2 incubator for 24 h. A total of ten microliters of CCK-8 solution was added to each well and then cultured in a 37 ℃ incubator for 2–4 h. The absorbance was detected at 490 nm using an enzyme labeling instrument, and the relative viability of cells treated with different drugs and different concentrations of samples was calculated.

6 Real-time PCR

RNA was extracted according to the rapid RNA extraction kit of the Shanghai Yishan Biotechnology Co., Ltd. (Shanghai, China). A total of 1000 ng mRNA was extracted according to the reverse transcription system (Table S1). The reverse transcription parameters were as follows: 37 °C for 15 min, 85 °C for 5 s, and kept at 4 °C until removed from the thermocycler. As shown in Table S2, complementary DNA was prepared for the PCR system according to the traditional three-step method. Primer information is shown in Table S3. At the end of the reaction, the relative expression levels of the genes in each group were analyzed using the $2^{-\Delta\Delta Ct}$ formula according to the cycle times of the internal reference gene, *β-actin*.

7 Western blotting

The total protein was extracted, and the protein concentration of each sample was determined according to the operation steps of the Solebo protein concentration determination kit. The samples were denatured at 100 °C for 8 min and stored in a refrigerator at −20 °C. After shaking and mixing, the protein sample was added to SDS gel, and the voltage was set to 80 V for 30 min, then changed to 120 V for 60 min. After the polyvinylidene fluoride (PVDF) membrane was activated with anhydrous methanol, it was transferred to the surface of the gel at a voltage of 100 V and a rotation time of 90 min. After film transfer, the sealing solution (5% skimmed milk powder) was sealed at room temperature for 1 h. After closure, the PVDF membrane was cleaned with Tris-buffered saline Tween-20 (TBST) containing 1‰ Tween-20 for 5 min. The antibodies (Anti-AXL and Anti-ACE2 antibodies were purchased from Beijing Bioss Biotechnology Co., Ltd. The anti-β-actin antibody was purchased from Shenzhen Aibokai Technology Co., Ltd. Anti-p16, Anti-p53, Anti-Rb, Anti-GAPDH antibodies were purchased from Cell Signaling Technology HRP- goat anti-mouse IgG and HRP-goat anti-rabbit IgG was purchased from Hangzhou Hua'an Biotechnology Co., Ltd.) was incubated overnight at 4 °C, washed with TBST three times for 5 min each, incubated with secondary antibodies at 37 °C for 1 h, washed with TBST three times, and then detected and analyzed.

8 Statistical analysis

All data are expressed as mean±standard deviation of three independent experiments. Statistical evaluation of differences between means of experimental groups was performed using two-tailed Student's *t*-test and one-way analysis of variance (ANOVA) analysis. Statistical significance was considered at * $P<0.05$, ** $P<0.01$ or *** $P<0.001$.

References

Jin WH, Tang H, Zhang JM, et al., 2020. Structural analysis of a novel sulfated galacto-fuco-xylo-glucurono-mannan from *Sargassum fusiforme* and its anti-lung cancer activity. *International Journal of Biological Macromolecules*, 149:450-458. https://doi.org/10.1016/j.ijbiomac.2020.01.275

Li J, Zhang Z, Tong T, 1995. The proliferative response and anti-oncogene expression in old 2BS cells after growth factor stimulation. *Mechanisms of Ageing and Development*, 80(1):25-34. https://doi.org/10.1016/0047-6374(94)01557-3

Mao GX, Zheng LD, Cao YB, et al., 2012. Antiaging effect of pine pollen in human diploid fibroblasts and in a mouse model induced by d-galactose. *Oxidative Medicine and Cellular Longevity*, 2012:750963. https://doi.org/10.1155/2012/750963

Zheng QH, Ma LW, Zhu WG, et al., 2006. $p21^{Waf1/Cip1}$ plays a critical role in modulating senescence through changes of DNA methylation. *Journal of Cellular Biochemistry*, 98(5):1230-1248. https://doi.org/10.1002/jcb.20838

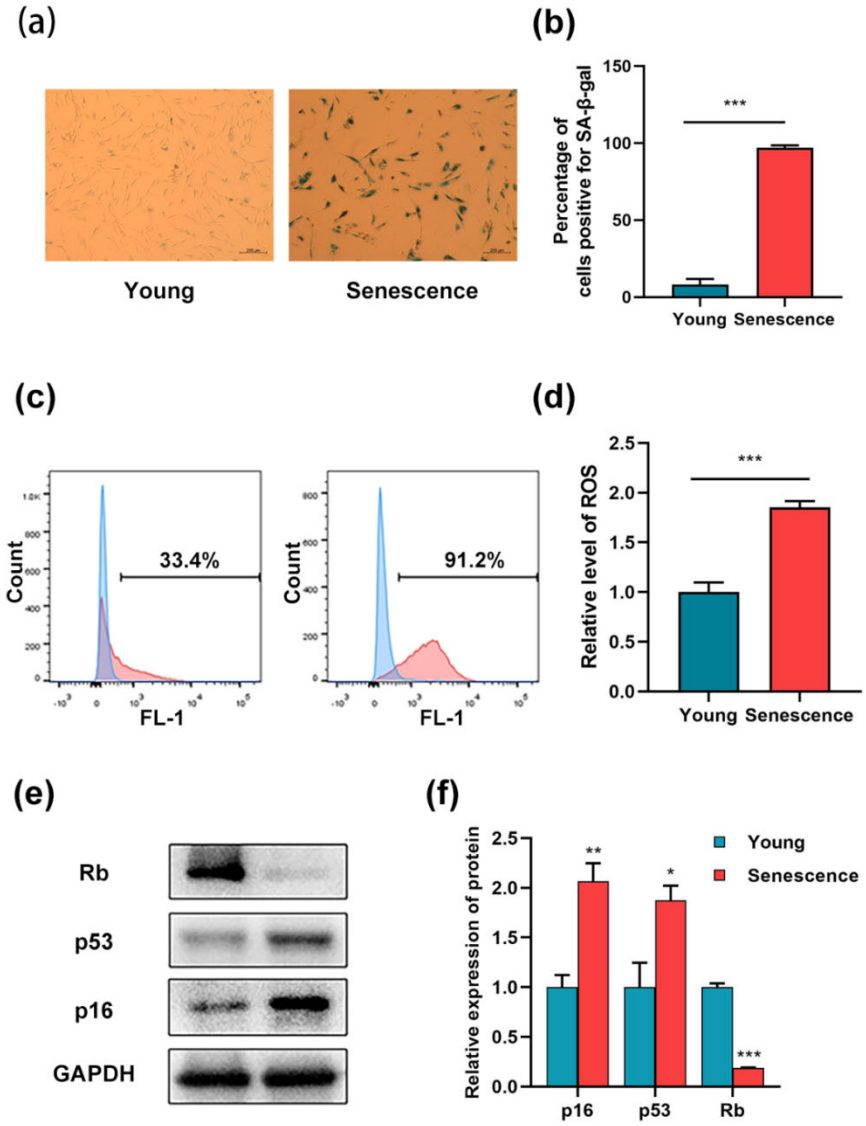

Fig. S1 Identification of 2BS replicative senescence model. (a) The results of SA-β-Gal staining in 2BS cells. The blue cells were positive (senescent) cells. (b) The positive rate of SA-β-Gal staining in young and senescent cells showed a significant difference. (c) The levels of reactive oxygen species in young and senescent 2BS cells were detected by flow cytometry. Blue: control; Red: 2BS cells treated by the ROS detection kit, which was analyzed by FlowJovX.0.7 software. (d) The relative levels of reactive oxygen radicals in young and senescent cells. (e) Western blotting of senescence-related proteins in young and senescent cells; (f) The relative senescence-related proteins expression level of AXL was calculated according to the corresponding optical density obtained by Image J software, and the significant level was tested by t-test. * P<0.05, ** P<0.01, *** P<0.001, vs. Young.

Table S1 Reverse transcription system

Reagent	Mass/volume
RNA	1000 ng
ddH_2O	Add to16 μL
Reverse transcription master mix	4 μL
Total volume	20 μL

Table S2 PCR system

Reagent	Volume (μL)
Forward primer	0.4
Reverse primer	0.4
ddH2O	3.2
PCR premix buffer	5.0
cDNA	1.0
Total volume	10.0

Table S3 Primer

Primer	Primer order (5' to 3')
AXL-F	GTGGGCAACCCAGGGAATATC
AXL-R	GTACTGTCCCGTGTCGGAAAG
β-actin-F	AGGCCAACCGCGAGAAGATGACC
β-actin-R	GAAGTCCAGGGCGACGTAGC