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

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Coronavirus disease 2019 (COVID-19) is a global infectious disease that seriously endangers human life and health and affects normal social activities. Since the pandemic outbreak from December 2019 to February 2023, the total number of confirmed cases has exceeded 753 million, and the deaths caused by COVID-19 have reached 6.6 million (https://covid19.who.int; accessed on Feb. 16, 2023). Currently, supportive therapy is the primary treatment for patients, as some antiviral drugs have demonstrated significant side effects during clinical treatment. Although vaccination has a particular effect on the prevention of novel coronavirus infection and the emergence of severe diseases, the variation in the virus strain caused the vaccine's protective effect to be greatly weakened or lost. Therefore, there is an urgent need to identify safe and effective drugs to prevent and cure COVID-19. Relevant research and exploration can also provide a reference for drug screening with which to address similar infectious diseases and public health events in the future.

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) binds to the host receptor angiotensin-converting enzyme 2 (ACE2) through its spike glycoprotein, which mediates membrane fusion and viral entry. Although the role of ACE2 as an SARS-CoV-2 receptor is apparent, previous research has shown that the expression of ACE2 is inadequate in various human tissues, especially in the respiratory tract (Wang S et al., 2021). Therefore, other host receptors or co-receptors may promote SARS-CoV-2 entry into the respiratory cells (Lim et al., 2022). AXL is a member of the receptor tyrosine kinase family (AXL, TYRO3, and MER). Growth arrest-specific protein 6 (GAS6) is a high-affinity AXL ligand. Previous studies have shown that GAS6/AXL signaling is an essential pathway for tumor cell survival, proliferation, migration, and invasion, making AXL a potential target for tumor therapy (Feneyrolles et al., 2014; Barata and Rini, 2017). AXL is expressed in almost all human organs. In human lung and bronchial epithelial tissues and cells, the expression of AXL is much higher than that of ACE2 (Lukassen et al., 2020; Sungnak et al., 2020). Recent studies have found that *AXL* knockout can significantly reduce SARS-CoV-2 infection in H1299 lung cancer cells and human primary lung epithelial cells. The expression level of AXL in the bronchoalveolar lavage fluid of COVID-19 patients was positively correlated with the level of SARS-CoV-2

spike protein (Wang S et al., 2021). Therefore, AXL is another candidate receptor for SARS-CoV-2 invasion and could be a potential target for future clinical intervention strategies.

We collected previously published single-cell sequencing data of healthy people from the Gene Expression Omnibus (GEO) database (Reyfman et al., 2019) and performed information collation and mining. We grouped the data using an age cutoff of 55 years and analyzed the differences in gene expression between different age groups. The results showed that the *AXL* messenger RNA (mRNA) expression level in the lung cells of the younger age group (<55 years old) was significantly higher than that of the older age group (≥55 years old) (Fig. 1).

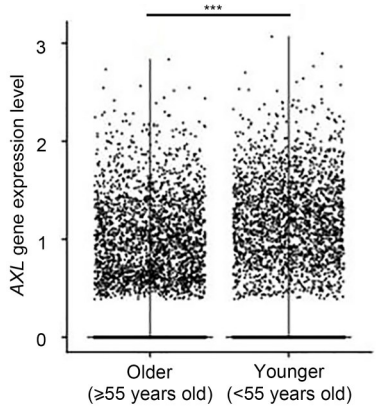

Fig. 1 Different expression of *AXL* in lung cells from younger (<55 years old) and older (≥55 years old) populations. * *P*<0.001.**

We constructed a senescence model using human embryonic lung diploid fibroblasts to explore whether AXL protein levels differ between young and senescent cells. Human embryonic lung diploid fibroblast (2BS) cells are not only an essential component of lung cells but are also recognized as a cell line for the study of senescence. 2BS cells were previously isolated from female fetal lung fibroblast tissue and have been fully characterized by the National Institute of Biological Products (Beijing, China). The cells are considered to be young at earlier than population doubling 30 (PD30) and fully senescent at PD55 or later. The cultured cells were split in ratio of 1:2 or 1:4 when the confluence of the culture was over 85%. The cumulative population doublings (CPDs) were calculated as $\log_2(D/D_0)$, where D and D_0 are defined as the density of cells at the time of harvesting and seeding, respectively (Tang et al., 1994; Li et al., 1995; Mao et al., 2010). The senescence model was established using 2BS cells to compare the protein expression of the novel coronavirus receptor AXL in young and senescent cells. Identification of the replicative senescent model was provided in Fig. S1.

Western blotting was used to detect the protein expression of AXL in young (PD30) and senescent (PD55) 2BS cells. The results showed that the expression of AXL in young cells was significantly higher than that in senescent cells (Fig. 2), which was consistent with the trend of expression levels obtained by bioinformatics analysis (Fig. 1). From the perspective of AXL as a novel coronavirus receptor, combined with the epidemiological investigation of the age distribution of infected people (Boehmer et al., 2020; Undurraga et al., 2021), it is speculated that the younger population may have a higher risk of infection. This suggests that, macroscopically, we should carry out epidemic prevention and remind younger people to attach importance to protection to reduce infection and transmission among younger people with

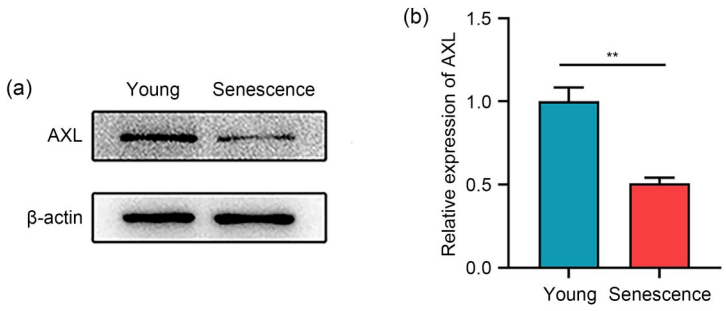

Fig. 2 Protein expression of AXL between young (PD30) and senescent (PD55) human embryonic lung diploid fibroblast (2BS) cells. (a) Representative western blotting images were acquired from three different experiments. (b) The relative expression level of AXL was calculated using ImageJ software. Data are expressed as mean±standard deviation (*n*=3). ** *P*< 0.01. PD: population doubling.

COVID-19. Based on the higher AXL expression in young 2BS cells, we selected young 2BS cells for further study as a potential model for artificial COVID-19 infection in vitro.

According to the literature on ancient Chinese medicine, brown algae have high edible and medicinal value. As a critical marine source of biomaterials, fucoidan sulfate is an ingredient in seaweed medicine and food homology. Fucoidan sulfate has biological activities such as tumor inhibition, immune regulation (Choi et al., 2005; Jin et al., 2020), and antiviral activity (Hoshino et al., 1998; Wang SY et al., 2021). In addition, fucoidan sulfate has high application value and good industrialization prospects in medicine and health. Fucoidan sulfate exhibits antiviral activity against herpes simplex virus (HSV), human papillomavirus (HPV), hepatitis B virus (HBV), and human immunodeficiency virus (HIV) (Béress et al., 1993; Damonte et al., 2004; Buck et al., 2006; Queiroz et al., 2008). Our previous studies have demonstrated that sulfated mannose oligosaccharides, tetrasaccharides, and hexasaccharides suppress human cytomegalovirus infection in vitro (Wang SY et al., 2021). It should be noted that the fucoidan sulfates exert the antiviral activity in vitro described above in the range of 10–1000 μg/mL. However, the regulatory effect of fucoidan sulfates on the expression of novel coronavirus receptor molecules has not yet been reported.

First, the biosafety of fucoidan sulfates was tested using a cell counting kit-8 (CCK-8) assay. Young (PD30) 2BS cells were pretreated with *Sargassum fusiforme* fucoidan sulfates at 100 and 500 μg/mL for 24 h. The cell viability was not significantly affected at 100 or 500 μg/mL when compared to that of the control, indicating that the components of fucoidan sulfate from *S. fusiforme* would not be toxic to 2BS cells with concentrations lower than 500 μg/mL (Fig. 3a).

Next, the protein expression of AXL in young (PD30) 2BS cells treated with various fucoidan sulfates at an indicated concentration of 200 μg/mL was analyzed using western blotting (Fig. 3b). Among the tested compounds, SFW-3 showed an optimal effect. SFW-3 is an aqueous extraction of the *S. fusiforme* polysaccharide, and the structure and composition of the sample were reported in our previous work (Jin et al., 2020). The effect of SFW-3 on *AXL* mRNA expression in 2BS cells was in line with that of protein expression (Fig. 3c). The primers and reaction systems used in the experiment can be seen in the Tables S1–S3.

At the beginning of the pandemic, the young population was generally believed to be more susceptible to infection because of their heightened external engagements. This study preliminarily explained the phenomenon of higher infection rates among younger people based on the expression of AXL, a potential receptor for SARS-CoV-2 in different age groups. It is suggested that the younger population is not resistant to infection, but mortality is lower, which may be due to the absence of an underlying condition, making recovery easier after infection. SARS-CoV-2 is transmitted through contaminants and droplets in unprotected close contact between infected and uninfected individuals. Symptomatic and asymptomatic patients are the primary sources of infection. The virus can also be transmitted through indirect contact: viral droplets contaminate the hands and then people come into contact with the mucous membranes of the mouth, nose, and eyes, resulting in an infection. Therefore, increased social activity represents a contributing factor to the higher incidence of infection among younger people. New opinions and suggestions on preventing and controlling the COVID-19 pandemic were proposed.

In this study, an elevated protein expression level of the AXL, a potential receptor for SARS-CoV-2, was proved in young 2BS cells, which indicated that it could serve as a potential model for artificial COVID-19 infection in vitro. A purified component of *S. fusiforme* fucoidan sulfate, SFW-3, was observed to inhibit AXL protein expression in young 2BS cells, which may at least partly occur due to down-regulation of the transcriptional level of *AXL*. SFW-3 may have an impact on the infection and development of SARS-CoV-2 through the GAS6/AXL signaling pathway (Morales et al., 2021); we will explore whether SFW-3 down-regulates the expression level of AXL protein by promoting the ubiquitination of AXL protein or by competitive binding to AXL receptors in our future work (Valverde, 2005).

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Data availability statement

All the authors declare that all the data in this paper are available.

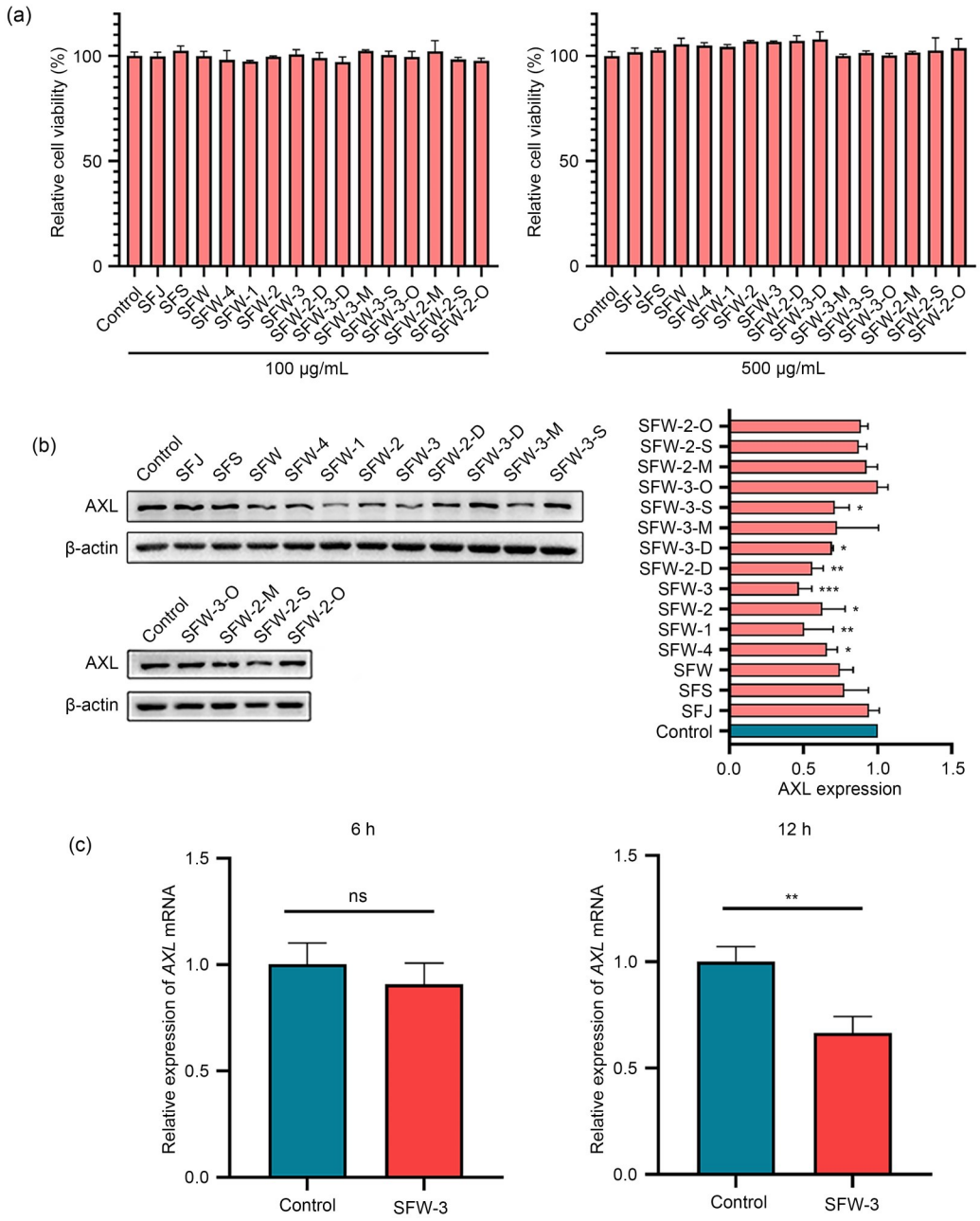

Fig. 3 Effects of *Sargassum fusiforme* fucoidan sulfates on cell viability and AXL expression in young (PD30) human embryonic lung diploid fibroblast (2BS) cells. (a) The effects of prepared samples on cell viability were assayed using cell counting kit-8 (CCK-8). Cells were treated with the different samples at indicated concentrations (100 and 500 μg/mL) for 24 h. The control group was set as 100%. (b) The inhibitive role of prepared samples in AXL protein expression in young 2BS cells. Cells were treated with indicated samples at 200 μg/mL for 24 h before being harvested for western blotting analysis. Representative western blotting images were acquired from three different experiments (left). The relative protein expression level of AXL was obtained by ImageJ software, with SFW-3 showing the optimal effect (right). (c) SFW-3 was selected to test its effect on the *AXL* messenger RNA (mRNA) expression. Cells were treated with 200 μg/mL SWF-3 for 6 and 12 h before being harvested for real-time polymerase chain reaction (PCR) assay. Data are expressed as mean±standard deviation (n=3). $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, vs. control; ns: not significant. SF refers to components derived from *S. fusiforme*, with W, S, and J representing aqueous, acidic, and alkaline extractions, respectively. Subsequent numbers denote various elution fractions, while M, S, and O indicate macro-molecules, small molecules, and oligosaccharides, respectively. D denotes small molecular weight fractions following oxidative degradation. PD: population doubling.

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Author contributions

Xuqiang ZHOU: conceptualization, methodology, data curation, formal analysis, and writing – original draft. Weihua JIN: conceptualization. Di JIANG: methodology and data curation. Yipeng XU: formal analysis. Sanying WANG: data curation. Xinna WU: writing – original draft. Yunchuang CHANG: visualization. Huili SU: data curation. Tianjun ZHU: visualization. Xiaogang XU: writing – review and editing. Genxiang MAO: supervision, funding acquisition, and writing – review and editing. All the authors have reviewed and approved the final version of the manuscript and agreed to be accountable for the content of the work.

Compliance with ethics guidelines

Xuqiang ZHOU, Weihua JIN, Di JIANG, Yipeng XU, Sanying WANG, Xinna WU, Yunchuang CHANG, Huili SU, Tianjun ZHU, Xiaogang XU, and Genxiang MAO declare that they have no conflict of interest.

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

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Supplementary information

Tables S1–S3; Fig. S1; Materials and methods