

## RESEARCH ARTICLE

# Polysaccharide from Polygonatum Inhibits the Proliferation of Prostate Cancer-Associated Fibroblasts Cells

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### Abstract

**Inhibition of cancer-associated fibroblasts (CAFs) may improve the efficacy of cancer therapy. Polysaccharide extracted from polygonatum can selectively inhibit the growth of prostate-CAFs ( $p < 0.001$ ) without inhibiting the growth of normal fibroblasts (NAFs). Polysaccharides from polygonatum stimulate autophagy of prostate-CAFs. 3-methyl-adenine(3-MA) is an autophagy inhibitor. 3-MA was added to prostate-CAFs with polysaccharide from polygonatum to determine whether autophagy plays an important role in the restrained effect. Finally, polysaccharide from polygonatum treatment significantly increased the activation of Beclin-1 and LC3, key autophagy proteins. Polysaccharide from polygonatum stimulates autophagy of prostate-CAFs and inhibits prostate-CAF growth, indicating that a novel anti-cancer strategy involves inhibiting the growth of prostate-CAFs.**

**Keywords:** Cancer associated fibroblasts - polysaccharide from polygonatum - autophagy - tumor - therapy

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### Introduction

Prostate cancer affects a gland in the male reproductive system. The cancer cells may spread from the prostate to other parts of the body, particularly bones and lymph nodes. Initially, patients may not have any symptoms. In the later stages, prostate cancer can result in difficulty urinating, blood in the urine, or pain in the pelvis or back when urinating. Prostate cancer is the most common noncutaneous cancer diagnosed among American men and the second leading cause of cancer death. Known risk factors for prostate cancer are increased age, African American race, and positive family history of the disease. As a result, developing adequate therapy for prostate cancer is an important medical goal. One of the most frequently used modalities for treating locally advanced prostate cancer, in addition to radical prostatectomy (RP) and radiation therapy (RT), is androgen-deprivation therapy (ADT).<sup>2</sup> The endocrine therapy used to treat prostate cancer aims to eliminate the androgenic activity in the prostatic tissue. Polygonatum, belonging to Liliaceae polygonatum, is a dried rhizome of perennial herb that has been used in traditional Chinese medicine. Polysaccharides from polygonatum exhibit strong anti-cancer activities. In the tumor microenvironment, fibroblasts are one of the most abundant cell types. CAFs promote tumor progression through producing an

inflammatory microenvironment that supports cancer cells. Cancer-associated fibroblasts (CAFs), a predominant stromal cell type in the tumor microenvironment, promote tumor growth and metastasis, induce tumor fibrosis and alter the composition and physiochemical properties of the extracellular matrix. In response to fast tumor growth, they create growth-induced solid stress, impeding the penetration of both macromolecules and nanomedicines. Preventing or inhibiting CAFs can in turn inhibit tumor growth, which could be used for a new strategy for treating cancer. Autophagy is an evolutionarily conserved process in which a portion of the cytoplasm is sequestered in a double-membrane enclosed autophagosome and then degraded upon fusion with a lysosome. Autophagy is a tightly regulated system in which the endogenous cellular protein aggregates, and damaged organelles are degraded via the lysosomal pathway. Recent studies suggest that autophagy plays a significant role in cancer progression and could be a target for treatment. In this study, we investigated the effect of polysaccharide from polygonatum on prostate-CAFs as well as examined the effect of polysaccharide from polygonatum on the autophagy of prostate-CAFs. The findings of this study support a novel mechanism for the anti-tumor activity of polysaccharides and indicate that polysaccharide from polygonatum is a potential candidate for anti-tumor therapy.

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## Materials and Methods

### Preparation of polysaccharide from polygonatum

Polysaccharide from polygonatum was extracted with hot water to obtain a total water soluble polysaccharide extract, and it was precipitated with 60% ethanol to obtain. The polysaccharide precipitate was redissolved in Dulbecco's modified Eagle's medium (DMEM) media at concentrations of 3mg/ml. The compound was stored at -20°C until further use.

### Cell culture

CAFs (PF179T-CAF, isolated from a prostatectomy specimen, marginal to the prostate tumor, hTERT immortalized; designated PF179; 179, patient number, Department of Urology, University of Innsbruck, Innsbruck Austria) and NAFs (isolated from a prostatectomy specimen, normal tissue of prostate) were provided by Prof. Ju Zhang at College of Life sciences, Nankai university. All these cell lines were authenticated by the suppliers and passaged in the laboratory for fewer than 3 months after resuscitation. CAFs and NAFs were maintained in a 37 °C/5% CO<sub>2</sub> humidified chamber in Dulbecco's modified Eagle's medium (DMEM) media (WISENT, Canada) supplemented with 10% FBS (WISENT, Canada).

### Western blot analysis

Proteins were extracted using RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.0% Nonidet P-40, 0.5% (w/v) sodium deoxy-cholate, 0.1% (w/v) SDS, and 1 mM EDTA] and quantified with the Bio-Rad Protein Assay (Bio-Rad Laboratories). Sixty micrograms of the total protein extract of each sample was separated by 15% (LC3) or 10% (Beclin-1) sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted on polyvinylidene fluoride membrane (Mini Trans-Blot, Bio-Rad Laboratories). The transferred membranes were probed with rabbit polyclonal antibody against LC3 at a 1:500 dilution (Bioss Inc) and with rabbit polyclonal antibody against Beclin-1 at a dilution ratio of 1:600 (Bioss Inc) at 4°C overnight. A goat anti-rabbit horseradish peroxidase conjugated secondary antibody (1:4000, KPL, Baltimore, MD, USA) was added. Membranes were developed with ECL (Amersham Biosciences, Little Chalfont, UK). Polyclonal antibody against  $\beta$ -actin was used as a loading control (Sigma-Aldrich).

### Cell survival assay (MTT)

The effect of polysaccharide derived from polygonatum on cell growth was determined with the MTT Cell Proliferation and Cytotoxicity Assay Kit. Prostate-CAFs were seeded into 96-well plates at an equal density of  $2 \times 10^5$  to  $5 \times 10^5$ /mL in cell culture medium and treated with different concentrations of polysaccharide from polygonatum (2  $\mu$ g/ml, 10  $\mu$ g/ml, 50  $\mu$ g/ml, 250  $\mu$ g/ml, and 1250  $\mu$ g/ml) for 48 h. The MTT Cell Proliferation and Cytotoxicity Assay Kit solution was added to each well at a volume of 10  $\mu$ L and then incubated for 4 h. Then, the formazan lysis solution [5% 2-Methyl-1-propanol, 10% SDS, 0.012 mol/L HCL] was added. After 4 h, the cell

viability was determined by measuring the absorbance at 570 nm with a microplate reader (BIO-RAD Laboratories, Philadelphia, PA, USA). All cell survival assays were performed in triplicate and repeated in 3 independent experiments.

### MDC staining

The autophagy level is characterized by the development of autophagic vacuoles. Prostate-CAFs were seeded into 6-well plates at an equal density of 5000 cells per well in cell culture medium and treated with different concentrations of polysaccharide from polygonatum (50  $\mu$ g/ml, 250  $\mu$ g/ml, and 1250  $\mu$ g/ml) for 4 h. Monodansylcadaverine (MDC) has been proposed as a tracer for autophagic vacuoles. The cells were then stained with 50  $\mu$ M MDC at 37°C for 1 hr. After incubation, the cells were fixed for 15 min with ice-cold 4% paraformaldehyde at 4°C. After three washes with 0.1 M PBS, and 1% PFA (150  $\mu$ L) was added to each well to fix the cells, which were then examined using a Nikon ECLIPSE Ti fluorescence microscope (Nikon, Tokyo, Japan).

### Cell survival assay (CCK)

The effect of polysaccharide from polygonatum on cell growth was determined using a Cell Counting Kit-8 (Zoman Biotechnology Co. Ltd. Beijing, China) according to the manufacturer's instructions. Cells were seeded into 96-well plates at density of  $2 \times 10^5$ /mL in cell culture medium and then treated with different concentrations of polysaccharide from polygonatum (2  $\mu$ g/ml, 10  $\mu$ g/ml, 50  $\mu$ g/ml, 250  $\mu$ g/ml, and 1250  $\mu$ g/ml) for 48 h. The CCK-8 reagent was added into each well at a volume of 10  $\mu$ L and then incubated for an additional 4 hrs. The cell viability was determined by measuring the absorbance at 450 nm using a microplate reader (BIO-RAD Laboratories, Philadelphia, PA, USA). All cell survival assays were performed in triplicate and repeated in 3 independent experiments.

## Results

### Polysaccharide from polygonatum inhibited prostate-CAF growth

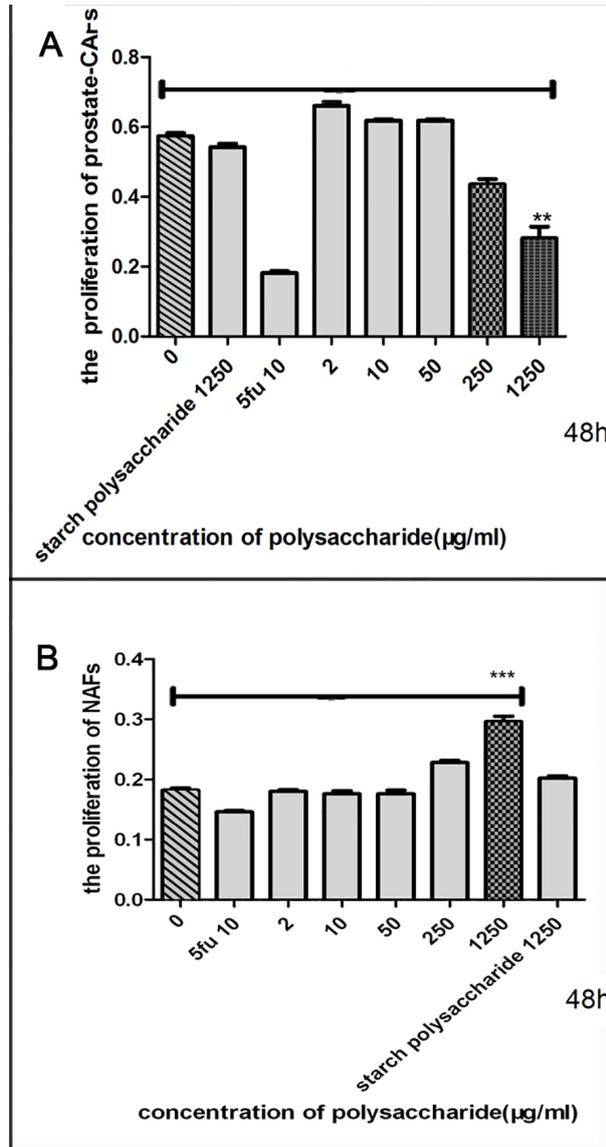
To evaluate whether polysaccharide from polygonatum can inhibit the growth of CAFs, we first examined its effect on the growth of prostate-CAFs. We used fluorouracil (5Fu) as a positive control and starch polysaccharide as a negative control. Prostate-CAFs were exposed to various concentrations (2  $\mu$ g/ml, 10  $\mu$ g/ml, 50  $\mu$ g/ml, 250  $\mu$ g/ml, and 1250  $\mu$ g/ml) of polysaccharide from polygonatum for 48 h. As shown in Figure 1(A), polysaccharide from polygonatum can inhibit the growth of prostate-CAFs. Following exposure to different concentrations of polysaccharide from polygonatum (250  $\mu$ g/ml and 1250  $\mu$ g/ml), the growth of prostate-CAFs was significantly inhibited (Figure 1A). Figure 1A shows the significant ( $p < 0.001$ ) increase in the relative inhibition levels for 48 h of treatment with 250 and 1250  $\mu$ g/ml polysaccharide from polygonatum. With the extended response concentrations, the effect of polysaccharide from

polygonatum is increasingly obvious. At the same time, the starch polysaccharide (1250 µg/ml) did not inhibit the growth of prostate-CAFs. As a result, polysaccharide from polygonatum effectively inhibits the growth of prostate-CAFs.

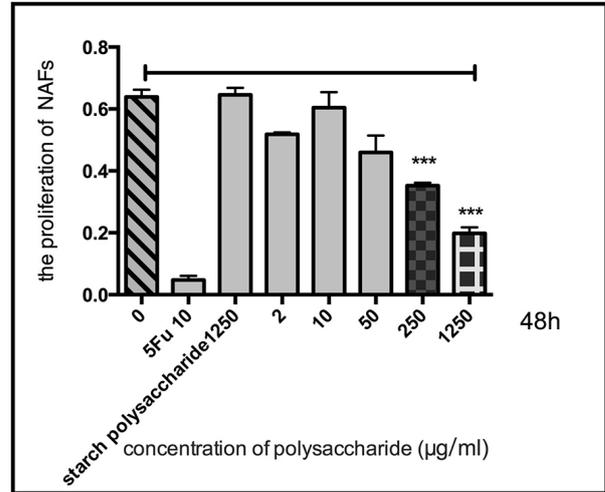
*The effect of different concentrations of polysaccharide from polygonatum on NAFs*

In addition to prostate-CAF inhibition, we evaluated the effect of different polysaccharide from polygonatum

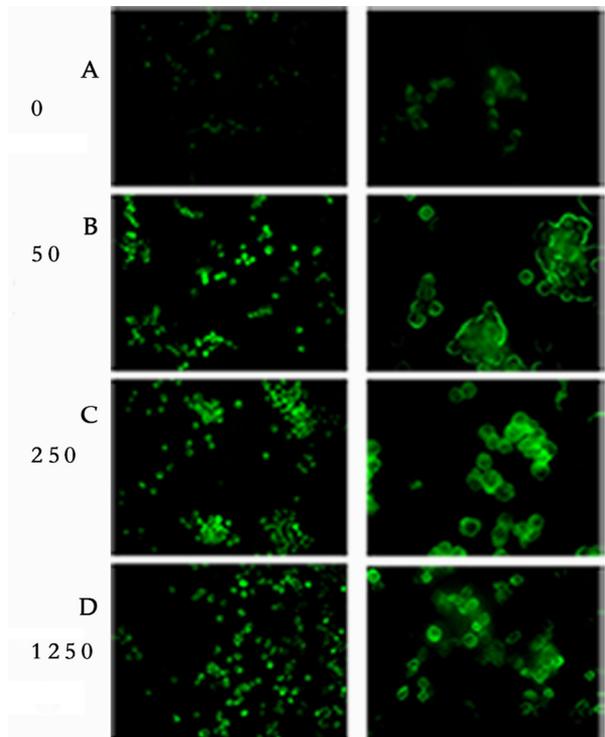
concentrations on NAFs. After 48 h treatment with polysaccharide from polygonatum, NAFs were analyzed using the MTT Cell Proliferation and Cytotoxicity Assay Kit. Interestingly, following exposure to different



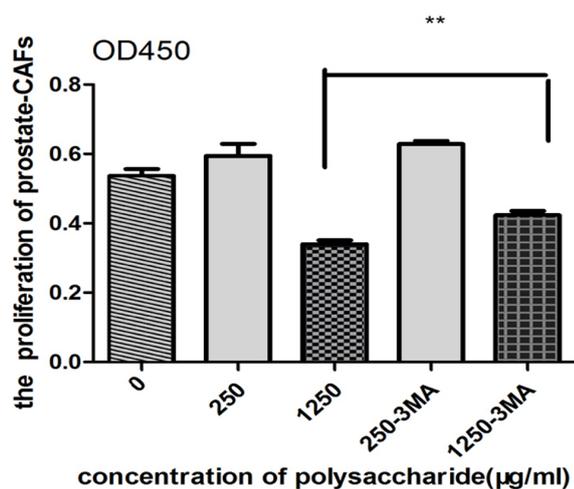
**Figure 1. The Effects of Different Concentrations of Polysaccharide from Polygonatum on NAFs and Prostate-CAFs.** (A) Prostate-CAFs were exposed to various concentrations (2 µg/ml, 10 µg/ml, 50 µg/ml, 250 µg/ml, and 1250 µg/ml) of polysaccharide from polygonatum for 48 h. Cell viability was detected using the MTT method. 5Fu was used as a positive control, and starch polysaccharide was used as a negative control. Prostate-CAFs were seeded in 96-well plates at a density of  $3 \times 10^5$ /ml. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . (B) NAFs were exposed to various concentrations (2 µg/ml, 10 µg/ml, 50 µg/ml, 250 µg/ml, and 1250 µg/ml) of polysaccharide from polygonatum for 48 h. The cell viability was detected using the MTT method. 5Fu was used as a positive control. NAFs were seeded in 96-well plates at a density of  $8 \times 10^5$ /ml. \*\*\* $p < 0.001$



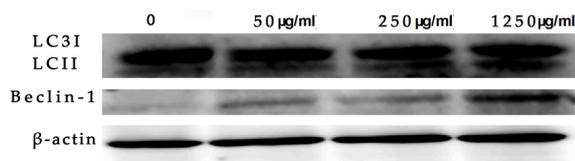
**Figure 2. The Effect of Polysaccharide from Polygonatum on CT26 Supernatant-exposed NAFs.** NAFs were cultured for 28 days with 30% culture supernatant from CT26 cells; then, NAFs were exposed to various concentrations (2 µg/ml, 10 µg/ml, 50 µg/ml, 250 µg/ml, and 1250 µg/ml) of polysaccharide from polygonatum for 48 h. The cell viability was detected using the MTT method. 5Fu was used as a positive control, and starch polysaccharide was used as a negative control. NAFs were seeded in 96-well plates in a density of  $8 \times 10^5$ /ml. \*\*\* $p < 0.001$



**Figure 3. Polysaccharide from Polygonatum Stimulates the Autophagy of Prostate-CAFs.** Prostate-CAFs were seeded in 6-well plates at a density of  $5 \times 10^5$ /mL and exposed to polysaccharide from polygonatum (50 µg/ml, 250 µg/ml and 1250 µg/ml) for 4 h. Then, the autophagy of prostate-CAFs was determined by MDC staining. (original magnification 100 and 200)



**Figure 4. The Effect of Different Concentrations of Polygonatum Polysaccharide on Prostate-CAFs that were Simultaneously Treated with 3-MA.** Prostate-CAFs were exposed to various concentrations (2 µg/ml, 10 µg/ml, 50 µg/ml, 250 µg/ml, 1250 µg/ml) of polysaccharide from polygonatum alone as well as at concentrations of 250 µg/ml and 1250 µg/ml with 3-MA (300 µg/ml) for 48 h. The cell viability was detected using a Cell Counting Kit-8 assay. 5Fu was used as a positive control, and starch polysaccharide was used as an negative control. Prostate-CAFs were seeded in 96-well plates at a density of  $2 \times 10^5$ /ml. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$



**Figure 5. Effect of Polysaccharide from Polygonatum on the Expression of Autophagy-related Proteins.** Prostate-CAFs were seeded in 6-well plates at a density of  $1 \times 10^6$ /mL, and they were exposed to various concentrations (50 µg/ml, 250 µg/ml, and 1250 µg/ml) of polysaccharide from polygonatum for 4 h. The expression of LC II/LC3I and Beclin-1 was determined by Western blot analysis. β-actin was used as an equal loading control for the samples

concentrations of polysaccharide from polygonatum (250 µg/ml and 1250 µg/ml), the growth of NAFs increased ( $p < 0.001$ ) (Figure 1B). At the same time, the inhibition of prostate-CAF growth was obvious. Together, these results indicated that polysaccharide derived from polygonatum only inhibited the proliferation of prostate-CAFs; it did not inhibit the growth of normal fibroblasts. Thus, polysaccharide from polygonatum had a significant inhibitory effect on prostate-CAFs, but it had not such effect on NAFs.

#### *Polysaccharide from polygonatum selectively inhibits CAFs induced from NAFs in vitro*

To test whether polysaccharide from polygonatum can selectively inhibit the growth of prostate-CAFs, NAFs were exposed to 30% culture supernatant from CT26 cells for 28 days. We evaluated whether polysaccharide from polygonatum can inhibit the growth of NAFs after culturing in a tumor. Figure 2 shows the significant ( $p < 0.001$ ) increase in the relative inhibition level with 48 h

of 250 and 1250 µg/ml polysaccharide from polygonatum treatment. As shown in Figure 2, polysaccharide from polygonatum inhibited the proliferation of CT26 supernatant-exposed NAFs. The results indicated that polysaccharide derived from polygonatum might selectively inhibit prostate-CAFs.

#### *Polysaccharide from polygonatum stimulates the autophagy of prostate-CAFs*

Autophagy helps maintain homeostasis, and recent studies have demonstrated that autophagy is involved in development, differentiation, aging and cell death. To evaluate whether the autophagy is involved in the effect of polysaccharide from polygonatum on prostate-CAF, we investigated the effect of polysaccharide from polygonatum on prostate-CAF autophagy. Prostate-CAFs were seeded in 6-well plates at a density of  $5 \times 10^5$ /mL, and they were exposed to polysaccharide from polygonatum (50 µg/ml, 250 µg/ml and 1250 µg/ml) for 4 h. The autophagy of prostate-CAFs was determined using MDC staining. The level of autophagy is characterized by the development of autophagic vacuoles. Monodansylcadaverine (MDC) has been proposed as a tracer for autophagic vacuoles. As shown in Fig. 3, there is a obvious difference in the autophagy with polysaccharide from the polygonatum and control groups (Figure 3 A, B, C, D). Our results demonstrated that polysaccharide from polygonatum stimulates prostate-CAF autophagy.

#### *Autophagy plays an important role in inhibiting the proliferation of prostate-CAFs*

To further explore whether autophagy plays an important role in inhibiting the proliferation of prostate-CAFs, we treated prostate-CAFs with polysaccharide from polygonatum (250 µg/ml and 1250 µg/ml) with 3-MA (300µg/ml) or polysaccharide from polygonatum alone. We analyzed the inhibition using a Cell Counting Kit-8 assay. 3-MA (3-methyl-adenine) is an autophagy inhibitor. 3-methyl-adenine (3-MA) can specifically inhibit the formation of autophagic vacuoles. After treatment with 3-MA, the proliferation of prostate-CAFs that were exposed to polysaccharide from polygonatum was up-regulated. A Cell Counting Kit-8 assay was performed to determine whether autophagy plays an important role in reversing survival after treatment with polysaccharide from polygonatum. As shown in Figure 4, after treatment with 3-MA, the reversal rate was 25% for 1250 µg/ml polysaccharide from polygonatum (Figure 4). As a result, autophagy plays an important role in inhibiting the proliferation of prostate-CAFs.

#### *Polysaccharide from polygonatum stimulates the expression of autophagy-related proteins*

To further clarify the mechanism of how polysaccharide from polygonatum stimulates the autophagy of prostate-CAFs, we examined autophagy-related proteins (LC3I II and Beclin-1). The mammalian autophagy gene Beclin-1, through interacting with class III phosphatidylinositol-3-phosphate kinase (PI3kIII/ Vps34), regulates autophagy. The yeast ATG-8 mammalian homolog microtubule-associated protein 1 light chain 3 (LC3)

is essential for final autophagosome formation. LC3 exists in two forms, a cytosolic form (LC3-I) and a lipid phosphatidylethanolamine-conjugated form (LC3-II). LC3-II is inserted into both the inner and outer membranes of the growing autophagosome. Therefore, LC3 can be used as a marker of autophagy by immunofluorescence studies, and LC3-I and LC3-II can be evaluated by Western blot analysis. The results demonstrated that polysaccharide from polygonatum stimulates the autophagy of prostate-CAFs through stimulating the expression of LC3 II and Beclin-1. Based on Figure 5, after the treatment with (50 µg/ml, 250 µg/ml, or 1250 µg/ml) polysaccharide from polygonatum, LC3 II and Beclin-1 were up-regulated. At the same time, β-actin was not up-regulated (Figure 5).

## Discussion

Early results indicated that polysaccharide derived from polygonatum has an obvious anti-tumor effect on H22 tumor-bearing mice. The mechanism may be related to regulation of the cell cycle distribution, arresting the tumor cells in the G0/G1 phase, inhibiting cell proliferation and activating the caspase system to induce apoptosis. Recently, results have suggested that polysaccharides from polygonatum have strong anti-cancer activity. However, to the best of our knowledge, there are no data indicating that polysaccharides affect the tumor microenvironment and inhibit tumor cell proliferation. Androgen deprivation therapy (ADT) for prostate cancer is one of the most effective known systemic palliative treatments for solid tumors. Moreover, it had been posited that polysaccharides affect cancer. We found that polysaccharide derived from polygonatum may stimulate prostate-CAF autophagy to selectively inhibit the proliferation of prostate-CAFs and inhibit prostate cancer proliferation. This finding may have implications for anti-cancer therapy.

In conclusion, Our results suggest that polysaccharide from polygonatum may stimulate prostate-CAF autophagy and inhibit the proliferation of prostate-CAFs, which is important for prostate cancer therapy.

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S.H., G.Q., M.H. contributed equally to this work. The authors thank Professor Ju Zhang from Nankai University for providing PF179T-CAF cell line.

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