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Original Paper

Fucoidan Inhibits the Proliferation of Leiomyoma Cells and Decreases Extracellular Matrix-Associated Protein Expression

Hsin-Yuan Chen[a] Tsui-Chin Huang[b,c] Li-Chun Lin[b] Tzong-Ming Shieh[d] Chi-Hao Wu[e] Kai-Lee Wang[f] Yong-Han Hong[g] Shih-Min Hsia[a,h,i,j]

[a]School of Nutrition and Health Sciences, College of Nutrition, Taipei Medical University, Taipei, [b]PhD Program for Cancer Biology and Drug Discovery, College of Medical Science and Technology, Taipei Medical University and Academia Sinica, Taipei, [c]TMU Research Center of Cancer Translational Medicine, Taipei Medical University, Taipei, [d]Department of Dental Hygiene, College of Health Care, China Medical University, Taichung, [e]Department of Human Development and Family Studies, National Taiwan Normal University, Taipei, [f]Department of Nursing, Ching Kuo Institute of Managemnet and Health, Keelung City, [g]Department of Nutrition, I-Shou University, Kaohsiung City, [h]Graduate Institute of Metabolism and Obesity Sciences, College of Nutrition, Taipei Medical University, Taipei, [i]School of Food and Safety, Taipei Medical University, Taipei, [j]Nutrition Research Center, Taipei Medical University Hospital, Taipei, Taiwan

Key Words
Uterine leiomyoma • Fucoida • Transforming growth factor beta • Extracellular matrix • ELT-3-LUC • Xenograft model

Abstract
Background/Aims: Uterine leiomyomas (ULs) are benign uterine tumors, and the most notable pathophysiologic feature of ULs is excessive accumulation of extracellular matrix (ECM). Fucoidan is a polysaccharide extracted from brown seaweeds that has a wide range of pharmacological properties, including anti-fibrotic effects. We aimed to study the effect of fucoidan on the growth of ULs activated by transforming growth factor beta (TGFβ). ***Methods:*** We used ELT-3 (Eker rat leiomyoma tumor-derived cells) and HUtSMC (human uterine smooth muscle cells) as *in vitro* models. Cell viability was determined by the MTT assay. Cell colony formation was stained using crystal violet. The side population, cell cycle and apoptosis were analyzed using flow cytometry. Protein expression was assayed by western blot analysis. We also conducted *in vivo* experiments to confirm the inhibitory effects of fucoidan in nude mouse xenograft models. Tumor tissues were assayed by immunohistochemistry analysis. ***Results:*** In our study, fucoidan caused a 50% growth inhibition using a dose of 0.5 mg/ml and decreased the stem cell activity after 48 h. In addition, fucoidan induced sub-G1 cell cycle arrest and apoptosis. Fucoidan down-regulated fibronectin, vimentin, α-SMA and the COL1A1 protein

Shih-Min Hsia
School of Nutrition and Health Sciences, Taipei Medical University,
250, Wu-Hsing Street, Taipei 11031 (Taiwan)
Tel. +886 2 7361661 6558, E-Mail bryanhsia@tmu.edu.tw

levels in TGFβ3-induced ELT-3 cells. In the cellular mechanism, fucoidan abrogated TGFβ3-induced levels of p-Smad2 and p-ERK1/2, as well as β-catenin translocation into the nucleus. Furthermore, fucoidan suppressed xenograft tumor growth *in vivo*. ***Conclusion:*** Fucoidan displays anti-proliferation and anti-fibrotic effects and exerts protective effects against ULs development.

Introduction

Benign uterine leiomyomas (ULs), also known as myomas or fibroids, have received attention as a female disease. ULs are the most common tumor of the uterus, affecting one in fourth women of reproductive age [1], and occur in over 77% of women in the United States [2]. Most women with ULs are asymptomatic; however, 20% of ULs patients usually suffer from a reduced quality of life with symptoms such as abnormal uterine bleeding, pelvic pain, frequent urination, and infertility [3, 4]. Although the etiology remains unclear, genetic factors, cytokines, growth factors, steroid hormones (estrogens and progestogens) and/or their receptors, and excessive production of extracellular matrix (ECM) plays pivotal roles in the development of ULs [3, 5]. In addition, hypoxia can trigger stem cell proliferation during the initiation of fibrosis, leading to fibroids [6].

Under normal physiological conditions, the degradation of the ECM is precisely regulated [7]. However, when the cells do not respond correctly to the usual signals, pathological fibrosis occurs [8]. The ECM components have attracted increased attention regarding ULs growth, which include fibronectin, collagens, and proteoglycans (biglycan and fibromodulin) [9, 10]. Transforming growth factor beta (TGFβ) is one of the most important cytokines underlying the accumulation of ECM constituents in ULs, especially TGFβ3 [11].

Some patients suffer from leiomyoma, apart from hysterectomy, and most therapeutic treatments only provide temporary effectiveness [12]. Postoperative complications can be relieve by using conventional medical treatment, but the cost related to ULs is considerable. To ameliorate the influence of conventional medical treatment, many studies have demonstrated the chemopreventive and anticancer potential of dietary polyphenols, such as resveratrol [13], epigallocatechin gallate (EGCG) [14], green tea extract [15], and strawberry extract [16]. Therefore, the selection of natural extracts as adjuvants for chemotherapy is important.

Fucoidan is a complex series of sulfated polysaccharides found in the cell-wall matrix of brown seaweeds that contain high amounts of L-fucose and sulfate ester groups [17, 18]. The structure of fucoidan and its composition strongly depend on the extraction source (the type of algae) and the method of extraction, thereby affecting its effectiveness. Fucoidan extracted from different species has varied biological activities, including anti-coagulant [19], anti-oxidant [20], immunomodulatory [21], anti-inflammatory [22], antibacterial activity [23], and anti-obesity [24, 25] properties. Alternatively, different processing methods are also important factors that influence the molecular conformational changes. Purified fucoidan preparation (PFP) has a higher cytotoxic effect due to the higher sulfate content compared with crude fucoidan preparation (CFP) [26]. Lowering the molecular weights (Mw) of fucoidan enhanced the anti-tumor activities by improving the binding properties of sulfate groups [27]. Furthermore, low-molecular-weight fucoidan (LMWF) prepared by gamma-irradiation not only avoided the removal of sulfate groups but also increased the cytotoxicity against cell transformation in cancer [28].

Recent *in vivo* and *in vitro* studies indicated that fucoidan is a potent anti-tumor agent in cancer cells (e.g., colon, liver, bladder and breast cancer) [29-35]. However, no study has reported the relationship of ULs and fucoidan. Therefore, in the present study, we aimed to investigate the inhibitory effects of fucoidan on ULs growth both *in vitro* and *in vivo*.

Materials and Methods

Compliance with ethical standards

All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the guidelines of the Taipei Medical University.

Cell culture

The Eker rat-derived uterine leiomyoma cells (ELT-3) were kindly provided by Dr. Lin-Hung Wei (Department of Oncology, National Taiwan University Hospital, Taipei, Taiwan). Eker rat-derived uterine leiomyoma cells transfected with luciferase reporter genes (ELT-3-LUC) were established in our laboratory previously. Primary human uterine smooth muscle cells (HUtSMC) were purchased from PromoCell (Heidelberg, Germany). ELT-3, ELT-3-LUC and HUtSMC cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, CAISSON Labs, UT, USA). Both cell lines were maintained in a medium containing 10% Fetal bovine serum (FBS) and 1% antibiotics (10, 000 units/ml penicillin, 10, 000 μg/ml streptomycin, and 25 μg/ml amphotericin with 8.5 g/L NaCl) and incubated at 37°C with 5% CO_2.

Reagents and antibodies

0.05% trypsin-ethylenediaminetetraacetic acid (EDTA, 1×), and antibiotic-antimycotic solution (100×) were purchased from CAISSON Labs (UT, USA). FBS, trypan blue, a bicinchoninic acid (BCA) protein assay kit, an electrochemiluminescence (ECL) immunoassay were purchased from Thermo Fisher Scientific (UT, USA). Bovine serum albumin (BSA) was purchased from BioShop (Burlington, Canada). Protease and phosphatase inhibitor cocktail tablets were purchased from Roche (Basel, Switzerland). Sodium bicarbonate, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Hoechst 33342, and verapamil were purchased from Sigma-Aldrich (MO, USA). VivoGlo™ Luciferin (*in vivo* Grade) was purchased from Promega (WI, USA). Matrigel® Basement Membrane Matrix was purchased from Corning (NY, USA). Zoletil® 50 was purchased from Virbac (Carros, France). Rompun® 20 (xylazine hydrochloride) was purchased from Bayer (Leverkusen, Germany). The following antibodies were used in this study (Table 1): anti-Bax, anti-Bcl-2, anti-p-Smad2, anti-Smad2/3, anti-p-ERK1/2, anti-ERK1/2, Histone H3, and anti-proliferating cell nuclear antigen (PCNA) were purchased from Cell Signal Technology (MA, USA); anti-vimentin, anti-α-SMA, anti-collagen type I alpha 1 (COL1A1), anti-β-catenin, and anti-β-actin were purchased from GeneTex (CA, USA); anti-cyclin-D1, anti-cyclin-dependent kinase 4 (CDK4), anti-fibronectin, anti-TβR2, and goat anti-rabbit/mouse antibody IgG were purchased from Abcam (Cambridge, UK).

Preparation of fucoidan and TGFβ3

The oligo-fucoidan powder from Laminaria japonica was kindly provided by Hi-Q Marine Biotech International Ltd (Taipei, Taiwan). A stock solution of 8 mg/ml was prepared in double-distilled H_2O (ddH_2O) and filtered using 0.22 μm sterile filters (Merck Millipore, MA, USA) and then stored at 4°C until use. For *in vitro* experiments, the final concentrations of fucoidan were prepared by diluting the stock with the cell culture medium. In addition, 50 mg/kg fucoidan was also prepared in ddH_2O and

Table 1. List of antibodies used in this study. WB: western blotting; IHC: immunohistochemistry; HRP: horseradish peroxidase

Antibody	Source (Catalogue No.)	Application	Dilutions
Primary antibody			
anti-Bax	Cell Signaling (#2772)	WB	1:1000
anti-Bcl-2	Cell Signaling (#2870)	WB	1:1000
anti-p-Smad2	Cell Signaling (#3104)	WB	1:1000
anti-Smad2/3	Cell Signaling (#5678)	WB	1:1000
anti-Phospho-p44/42 MAPK	Cell Signaling (#9101)	WB	1:1000
anti-p44/42 MAPK (Erk1/2)	Cell Signaling (#9102)	WB	1:1000
anti-Histone H3	Cell Signaling (#9715)	WB	1:10000
anti-PCNA	Cell Signaling (#13110)	IHC	1:1000
anti-β-catenin	Cell Signaling (#8480)	WB	1:1000
anti-vimentin	GeneTex (GTX100619)	WB	1:20000
anti-α-SMA	GeneTex (GTX100034)	WB/ IHC	1:1000/ 1:400
anti-COL1A1	GeneTex (GTX112731)	WB	1:500
anti-β-actin	GeneTex (GTX109639)	WB	1:20000
anti-TβR2	Abcam (Ab86838)	WB	1:1000
anti-cyclin-D1	Abcam (Ab134175)	WB	1:10000
anti-CDK4	Abcam (Ab3112)	WB	1:1000
anti-fibronectin	Abcam (Ab2413)	WB/ IHC	1:1000/1:200
Secondary antibody			
Goat Anti-Rabbit IgG H&L (HRP)	Abcam (Ab205718)	WB	1:10000
Goat Anti-Mouse IgG H&L (HRP)	Abcam (Ab205719)	WB	1:10000

used in *in vivo* experiments. The control groups were treated with a vehicle (ddH_2O) both *in vitro* and *in vivo*. The recombinant human TGFβ3 was purchased from PeproTech (Rehovot, Israel). A stock solution of 100 μg/ml was prepared in ddH_2O and then stored at -20°C until use. The final concentrations of TGFβ3 were prepared by diluting the stock with the cell culture medium.

Cell viability assay

To evaluate the cytotoxic effect of fucoidan, we used an MTT assay. The ELT-3 cells were seeded in 96-well plates (2×10^3 cells/well), cultured for 24 h, and treated with various concentrations of fucoidan (0, 0.1, 0.25, 0.5, 1 mg/ml) in fresh medium containing 1% FBS. The MTT solution (1 mg/ml) was added directly to each well (100 μL/well) for 4 h. The absorbance was measured on the Epoch Microplate Spectrophotometer (BioTek, VT, USA) at 570 nm, with a reference wavelength of > 630 nm.

Cell counting and colony formation assay

ELT-3 cells were seeded onto 6-well plates and treated with 0.5 mg/ml fucoidan and 10 ng/ml TGFβ3 in fresh medium containing 1% FBS. After 96 h, the cells were harvested in culture medium with 0.05% trypsin-EDTA solution. All of the cells and medium were collected at 4°C and spun down at 500 g centrifugation for 5 min. The cells were resuspended in 1 ml of medium, and 10 μl was used for cell counting in a hemocytometer with trypan blue staining to obtain the number of live and dead cells. In another hand, 0.5 mg/ml fucoidan and 10 ng/ml TGFβ3 were added to the wells for 96 h; the cells were then detached, reseeded onto a 10 cm^2 dish at a density of 2×10^3 cells per dish, and cultured with fresh medium. Cells were incubated at 37°C under 5% CO_2 for 5 days to allow the colonies to form (defined as containing 50 or more cells). Colonies were fixed in methanol and stained with 0.01% crystal violet for 20 min at room temperature. After removing the dye by washing, the colonies were photographed and then dissolved in DMSO with shaking for 3 h. Then, 0.1 ml of destain solution was added into the 96 wells and the absorbance was measured on the Epoch Microplate Spectrophotometer at 570 nm.

Side population (SP) isolation

To detect of the distribution of side population, ELT-3 cells were seeded at a density of 1×10^6 per dish and cultured for 24 h. The medium was replaced with fresh medium containing 1% serum with various concentrations of fucoidan (0, 0.5 and 1 mg/ml). After 48 h, the cells were dissociated with trypsin (0.05%), centrifuged at 1, 200 g for 5 min at 4°C, and resuspended in 1 ml of cold HBSS (Hanks' balanced salt solution) with 2% FBS. Hoechst 33342 (5 μg/ml) was then added to a density of 1×10^6 cells per tube. A parallel aliquot was stained with Hoechst 33342 dye in the presence of 50 μM verapamil. After incubation for 120-150 minutes and protection from light, the cells were centrifuged at 1, 200 g centrifugation for 5 min at 4°C and resuspended in 1 ml of cold HBSS solution with 2% FBS. Finally, stem cells were analyzed using a FACS flow sorter.

Flow cytometry analysis for the cell cycle distribution and apoptosis

To assess cell cycle progression, ELT-3 cells were seeded onto culture dishes and incubated for 24 h to allow for exponential growth. The cells were co-treated with 0.5 mg/ml fucoidan and 10 ng/ml TGFβ3 for 72 h. All of the cells were collected, 9 ml of 70% ethanol was added slowly, and the cells were stored at -20°C for at least 2 h. The cells were washed at least once with cold PBS and resuspended in 300-500 μl propidium iodide (PI)/Triton X-100 staining solution (2 mg DNAse-free RNAse A and 0.40 ml of 500 μg/ml PI was added to 10 ml of 0.1% (v/v) Triton X-100 in PBS). The cells were incubated for 30 min at 20°C. Fluorescence was measured with a FACSCalibur flow cytometer (BD, CA, USA) and cell cycle distribution analyzed using CellQuest and Modfit LT programs (BD, CA, USA). For the apoptosis analysis, a commercial fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (BD, CA, USA) was used. The ELT-3 cells were seeded in culture dishes and co-treated with 0.5 mg/ml fucoidan and 10 ng/ml TGFβ3 for 72 h. Then, the cells were stained with Annexin V-FITC and PI, incubated for 15 min at room temperature and protected from light. Apoptosis was analyzed using a FACSCalibur flow cytometer, and the results were analyzed using CellQuest software.

Western blot analysis

The lysates of tumor tissues were prepared in ice-cold lysis buffer containing a protease inhibitor cocktail. The proteins (30 μg) were boiled for 5 min, separated using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred electrophoretically to Immobilon-P polyvinylidene fluoride (PVDF) membranes for 150–180 min at 280 mA and 250 V. Then, the membranes were blocked with blocking buffer (5% (w/v) BSA in 1X TBST for 1 h at 25°C, and incubated for 8 h with primary antibodies (Table 1) at 4°C. The next day, the membranes were washed three times for 10 min each with TBST buffer, incubated for 1 h in blocking buffer with horseradish peroxidase (HRP)-conjugated secondary antibodies, and washed with TBST buffer three times for 10 min each. Finally, the bands were detected using ECL. The values were quantified and normalized to the internal control β-actin. Then, densitometric estimation was performed using ImageJ software. Each bar graph represents the mean± SD for at least three independent experiments.

Tumor xenografts in nude mice and experimental processes

In order to confirm the inhibitory effects of fucoidan was consistent with *in vivo* experiments, the nude mouse xenograft models was used. Five-week-old female Nude-Foxn1^{nu} mice (BioLASCO, Taipei, Taiwan) were housed under a 12 h light/12 h dark cycle in a pathogen-free environment with *ad libitum* access to food and water. Tumors were implanted by subcutaneous (s.c.) injection of ELT-3-LUC cells [1×10^6 cells suspended in 0.1 ml phosphate-buffered saline (PBS)/Matrigel solution for each mice] into the right flank of the mice. After the tumors reached a size of 50–100 mm^3, the mice were randomly assigned to two groups (n = 3 per group): one group received a daily oral gavage of fucoidan (50 mg/kg; treatment group) and the other group received a vehicle (ddH_2O; control group) every day for 42 days. The tumor volume was measured using calipers and calculated as $L \times W^2 \times 0.52$, where L is the length and W is the width. Tumor volumes and body weights were recorded until the animals were sacrificed by an i.p. injection of anesthetic mixture [1 ml Zoletil + 0.1 ml rompun + 3.9 ml normal saline (NS)]. Every week, the mice were administered an i.p. injection of luciferin (150 mg/kg body weight) and visualized using a Non-Invasive *In vivo* Imaging System (IVIS). At the end of the experiment, the tumors were formalin-fixed and stained with hematoxylin and eosin (H&E), PCNA, fibronectin, and α-SMA. All of the animal studies were conducted according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Taipei Medical University (IACUC Approval No. 2015–0447).

Immunohistochemistry analysis

To observe the localization of specific proteins were consistent with *in vivo* experiments, immunohistochemistry analysis was assayed. Tumor tissues were embedded and sliced at 2- or 6-μm thick by the animal experiment center of Taipei Medical University (Taipei, Taiwan). Tissue sections were stained by BIO-CHECK LABORATORIES LTD (Taipei, Taiwan). To analyze immunohistochemistry slides, five areas were photographed at 40 X magnifications (center, bottom, top, left and right regions) using an EVOS® microscope (Thermo Fisher Scientific, UT, USA) and the color of the PCNA, fibronrctin and α-SMA staining in the tissue sections was observed.

Statistical analysis

The data were presented as the mean ± standard deviation (SD), and the differences between the means were analyzed using the Sigma Plot version 12.5. For the comparison of two groups, Student's t-test was used. Means of more than two groups of data were compared using a one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) and Duncan's multiple-range post hoc test. The difference between two means was considered statistically significant when $p < 0.05$ and highly significant when $p < 0.001$.

Results

Cytotoxicity of fucoidan on leiomyoma cells and putative tumor-initiating cells

To evaluate the cytotoxic effect of fucoidan, ELT-3 were treated with fucoidan (0, 0.1, 0.25, 0.5, 1 mg/ml) for 24 and 48 h, and cell viability was measured using the MTT assay.

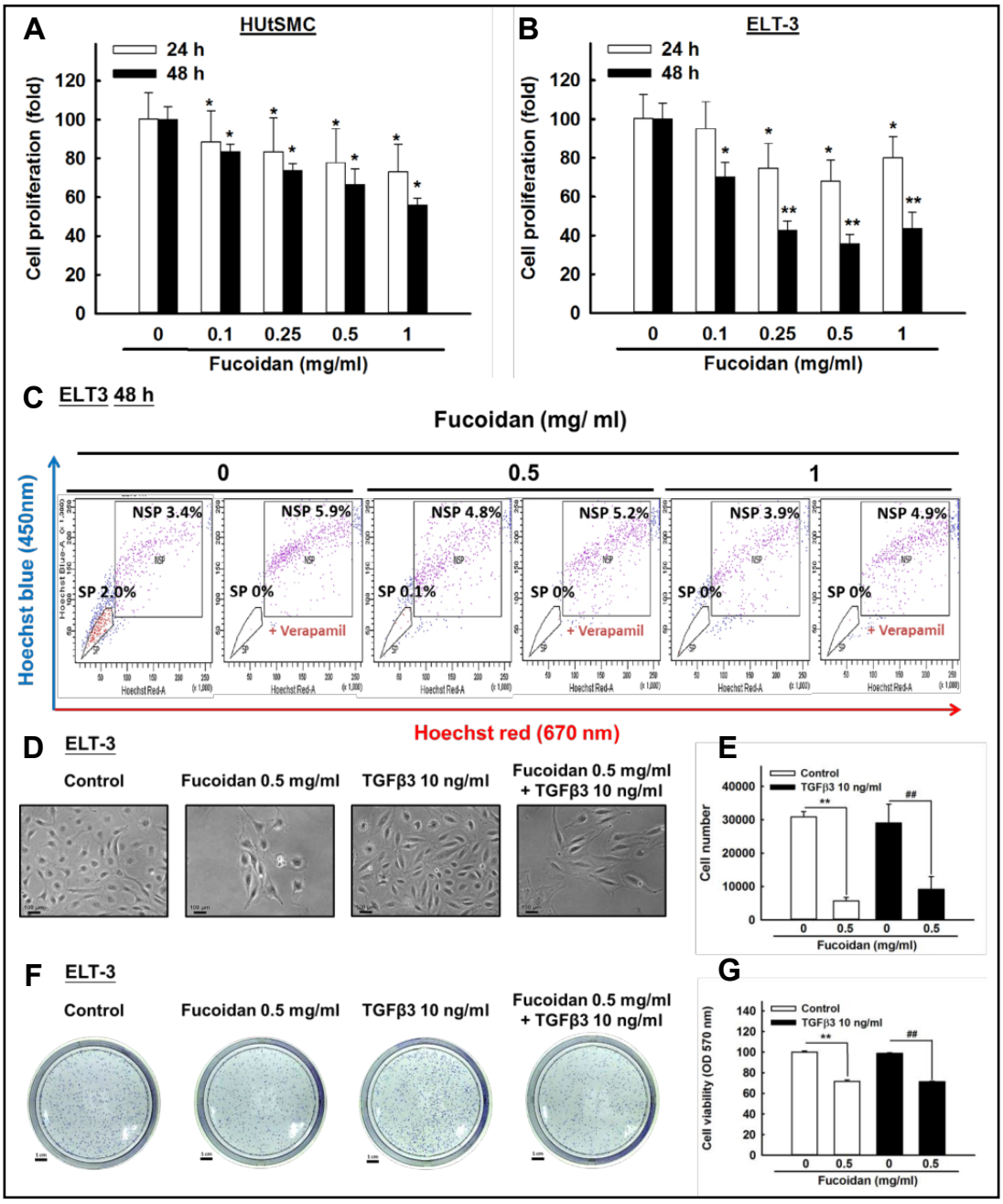

Fig. 1. Cytotoxicity of fucoidan on leiomyoma cell and putative tumor-initiating cells. (A) HUtSMC and (B) ELT-3 cells were exposed to the indicated concentration of fucoidan from 0.1 to 1 mg/ml follow by 24-48 h incubation. Cell proliferation was measured by MTT assay. (C) Analysis of side population (SP) distribution after fucoidan treatment (0, 0.5 and 1 mg/ml). Distribution of the SP and Non-SP (NSP) living cells isolated from ELT-3 leiomyoma cell fraction. SP cells represent 2% of total living cells stained with Hoechst 33342 (fucoidan 0 mg/ml group, left panel). Co-addition of 50 μM verapamil (inhibitor of ABC transporters) resulted in the disappearance of the SP fraction, negative control (each group, right panel) (n = 3). (D) and (E) ELT-3 cells were treated with 0.5 mg/ml fucoidan and TGFβ3 10 ng/ml follow by 96 h incubation, then cell number were counted by eye after trypan blue stain; scale bar = 100 μm. (F) and (G) Cell colony formation were stain by violet crystal, and measured at 570 nm; scale bar = 1 cm. The results are expressed as means ± SD of three independent experiments. Significances were calculated with one-way ANOVA analysis and Duncan's multiple-range post-test. $^{*}p < 0.05$ and $^{**}p < 0.001$ compared with control; $^{\#\#}p < 0.001$ compared with TGFβ3 10 ng/ml.

Fucoidan decreased the growth of uterine leiomyoma cells in a time-dependent manner (Fig. 1A and B), and we also calculated that the half maximal inhibitory concentration (IC_{50}) is 2 or 0.3 mg/ml for fucoidan in HUtSMC or ELT-3 cells, respectively. Based on the above results, we selected 0.5 mg/ml fucoidan as the concentration for the subsequent experiments.

To investigate whether leiomyomas have a population of stem cells that enable the repeatable enlargement of the uterus, we isolated putative leiomyoma stem cells from the ELT-3 leiomyoma cell fraction. Based on the side-population (SP) phenotype characteristic of the unique ability to efflux the hydrophobic fluorescent dye Hoechst 33342 via the ABC-transporter [36], we selected the putative leiomyoma stem cells and found that these cells represented 2.0% of the total living cell population (fucoidan 0 mg/ml group, left panel). However, we found that fucoidan decreased the number of selected stem cells for SP distribution (fucoidan 1 mg/ml group, left panel). In addition, separation of the SP cells was blocked by the addition of 50 M verapamil (negative control), an ABC transport protein inhibitor, and resulted in the disappearance of the SP fraction (each group, right panel) (Fig. 1C).

Inhibition of TGFβ3–induced cell growth in leiomyoma cells by fucoidan

According to a previous study, TGFβ is considered one of the key factors in the pathogenesis mechanism of uterine leiomyoma. To simulate the generated state of uterine leiomyoma, we also used TGFβ to stimulate ELT-3 cell growth; simultaneously, the cells were co-treated with fucoidan to inhibit the cell viability induced by TGFβ3 treatment. In Fig. 1D, after induction by TGFβ3 treatment, ELT-3 cells were spindle-shaped and had elongated nuclei for 96 h, but rounded floating cells were observed after 0.5 mg/ml fucoidan alone or co-treatment with 10 ng/ml TGFβ3. In addition, using the trypan blue exclusion test to examine the cell viability of ELT-3, as shown in Fig. 1E, 0.5 mg/ml fucoidan alone or co-treatment with 10 ng/ml TGFβ3 significantly reduced the number of ELT-3 cells for 96 h. Colony formation assays are often used to examine the long-term inhibitory effects on cell viability. We found that the colony formation number of ELT-3 was significantly decreased after fucoidan treatment compared with controls for 5 days. Similarly, when co-treated with TGFβ3, fucoidan treatment also significantly suppressed the colony formation number of ELT-3 compared with the TGFβ3 alone group (Fig. 1F and G).

Effects of fucoidan on cell cycle progression at sub-G1 phase and apoptosis-associated protein expression

To further investigate whether the cytotoxicity of fucoidan is associated with cell cycle arrest, we examined its effects on cell cycle progression and the results showed that 0.5 mg/ml fucoidan arrested the cell cycle at the sub-G1 checkpoint phase (Fig. 2A and C) and caused the protein expression of cyclin D1 was decreased in ELT-3 cells (Fig. 2D and F). In addition, when co-treated with TGFβ3, fucoidan treatment also increased the percentage of sub G1 phase (Fig. 2A and C) and caused cyclin D1 protein expression significantly decrease in ELT-3 cells (Fig. 2D and F) compared with the TGFβ3 alone group. Then, we investigated whether fucoidan induced apoptosis in leiomyoma cells using FACS analysis with Annexin V-FITC and PI double staining and the results showed that the percentage of apoptotic cells significantly increased from 3.9% to 24% in ELT-3 cells after treatment with 0.5 mg/ml fucoidan for 72 h (Fig. 2G and I). Similarly, when co-treated with TGFβ3, fucoidan treatment also significantly increased the percentage of apoptotic cells compared with the TGFβ3 alone group (Fig. 2G and I). In addition, the apoptosis-associated protein (Bax/Bcl-2 ratio) levels were increased in ELT-3 cells after 0.5 mg/ml fucoidan treatment (Fig. 2H and J). Our results demonstrated that fucoidan-induced apoptosis was mainly associated with an increase in the number of apoptotic cells (sub-G1 population).

Fucoidan ameliorates TGFβ3–induced extracellular matrix (ECM) accumulation in vitro

Numerous studies have shown that TGFβ can enhance excessive ECM production and storage in uterine leiomyoma. As western blot analysis showed, TGFβ3 treatment enhanced

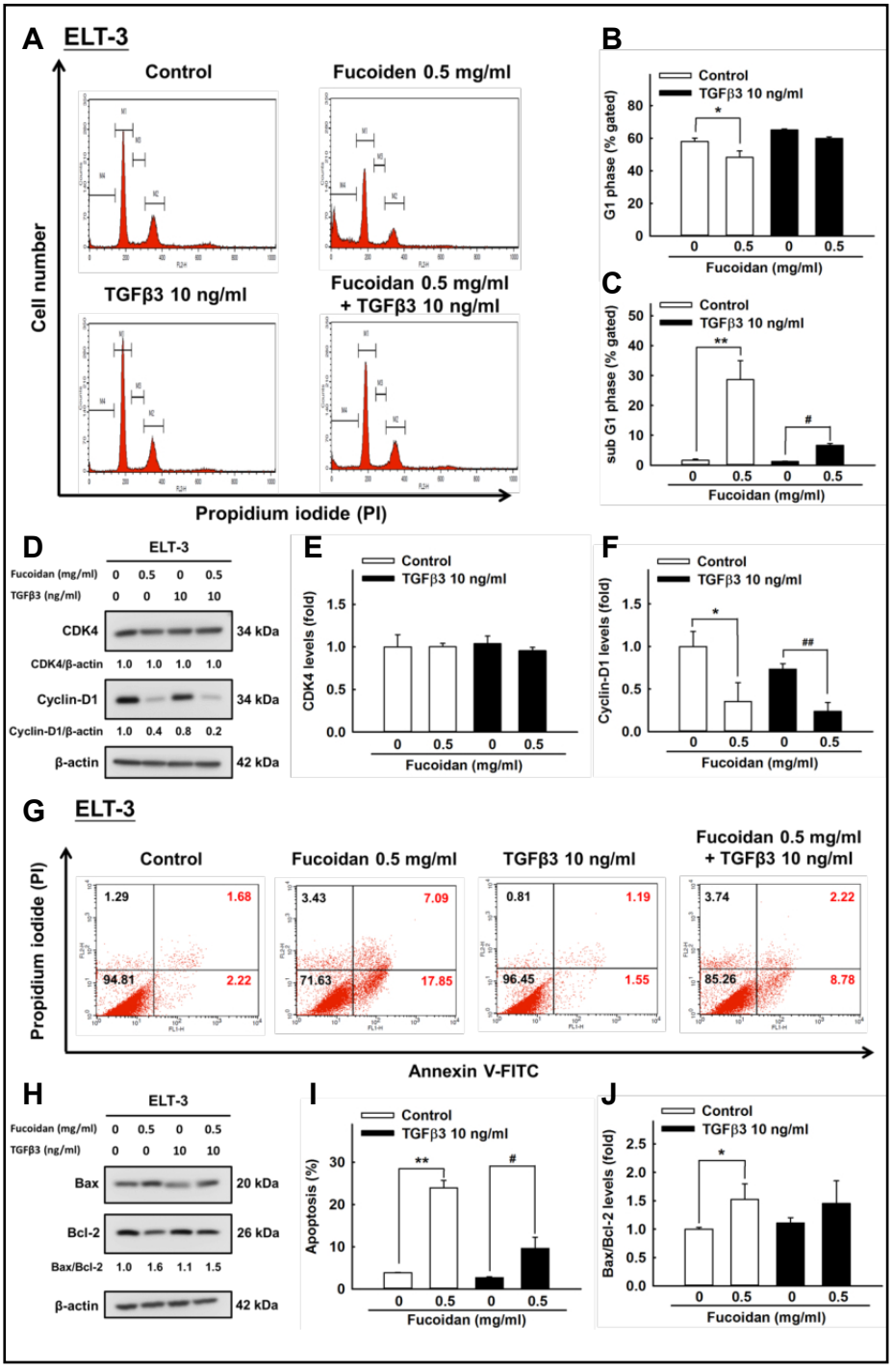

Fig. 2. Fucoidan induces sub-G1 cell cycle arrest and apoptosis in leiomyoma cell lines. Cells were plated in 100 mm diameter dishes at 2 x 10^5 cells in medium with 10% FBS until attach the plate bottom. ELT-3 cells were co-treated with 0.5 mg/ml fucoidan and TGFβ3 10 ng/ml for 72 h. (A) The cells were stained with propidium iodide (PI), and the histograms of cell cycle distribution was analyzed by flow cytometry. The vertical axis represents the cell numbers; the horizontal axis represents the PI stained strength. (B) and (C) The cell cycle distribution was shown in bar graph. The vertical numbers represents the cell population percentage in cell cycle G1 and sub-G1 phase, the horizontal number represents the dose of fucoidan. (G) ELT-3 cells were harvested and stained with Annexin V-fluorescein isothiocyanate (FITC) and PI, and cell apoptosis was analyzed using flow cytometry. (I) The percentage of apoptosis was shown in bar graph. (D) and (H) ELT-3 cell lysates were separated by SDS-PAGE and analyzed by western blot with the indicated antibodies. β-actin was used as a loading control. The values of the band intensity represent the densitometric estimation of each band normalized to β-actin. Protein quantification of CDK4 (E), cyclin-D1 (F), and Bax to Bcl-2 (J) expression in ELT-3 cells was shown in bar graph. Significances were calculated with one-way ANOVA analysis and Tukey's HSD post-test. $^{*}p < 0.05$ and $^{**}p < 0.001$ compared with control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.001$ compared with TGFβ3 10 ng/ml.

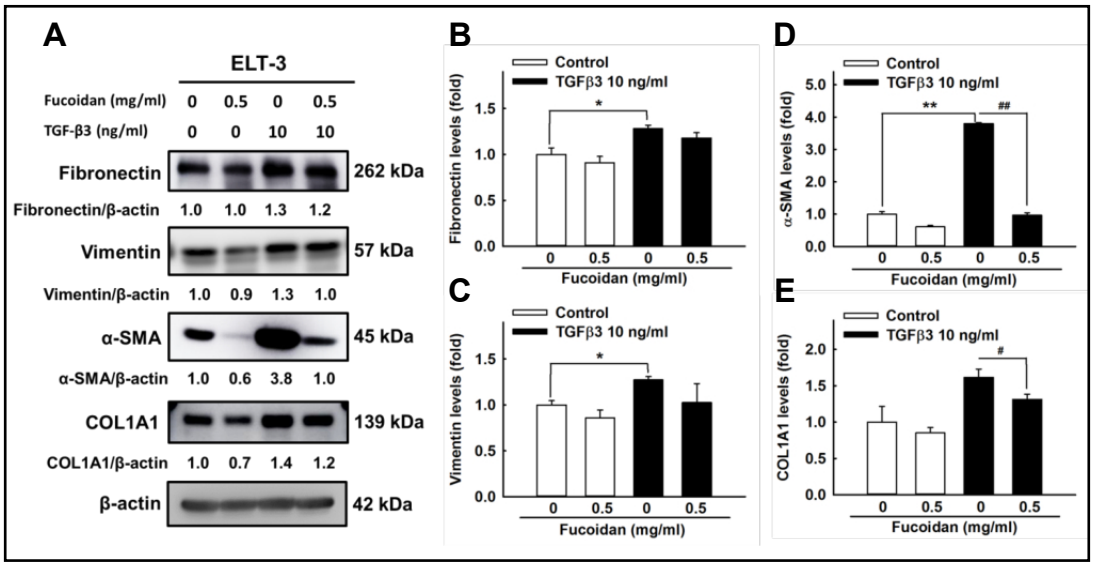

Fig. 3. Effect of fucoidan and TGFβ3 on protein expressions of ECM-related protein in ELT-3 cells. ELT-3 cells were co-treated with 0.5 mg/ml fucoidan and TGFβ3 10 ng/ml for 48 h. (A) ELT-3 cell lysates were separated by SDS-PAGE and analyzed by western blot with anti-fibronectin, vimentin, α-SMA, and COL1A1. β-actin was used as a loading control. The values of the band intensity represent the densitometric estimation of each band normalized to β-actin. Protein quantification of fibronectin (B), vimentin (C), α-SMA (D), and COL1A1 (E) expression in ELT-3 cells was shown in bar graph. Significances were calculated with one-way ANOVA analysis and Tukey's HSD post-test. $^{*}p < 0.05$ and $^{**}p < 0.001$ compared with control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.001$ compared with TGFβ3 10 ng/ml.

excessive deposition of ECM proteins, such as significantly enhanced the level of fibronectin, vimentin, and α-SMA, as well as up-regulating the expression of COL1A1 compared to controls. To investigate whether fucoidan decreased ECM accumulation in leiomyoma cells, we co-treated with fucoidan and TGFβ3 and found that fucoidan abrogated the protein expression of fibronectin, vimentin, α-SMA, and COL1A1 after induction by TGFβ3 treatment (Fig. 3A-E). These data demonstrated the potent inhibitory effect of fucoidan on ECM accumulation in leiomyoma *in vitro*.

Profibrotic response of TGFβ3 in ELT-3 cells is mediated by β-catenin, Smad and non-Smad ERK1/2 signaling pathways

To clarify whether fucoidan and TGFβ3 could affect the level of β-catenin in ELT-3 cells, western blot analysis was performed. We found that fucoidan reduced the level of β-catenin in the cytoplasm and therefore abrogated the TGFβ3-induced level of β-catenin translocation into the nucleus in ELT-3 cells (Fig. 4A-D). In addition, we also investigated whether fucoidan could regulate the mechanism under the TGFβ3-induced Smad pathway. As indicated in Fig. 4E and G, we found that TGFβ3 induced the expression of phosphorylated Smad2 after 4 h, whereas 0.5 mg/ml fucoidan abrogated TGFβ3-induced the phosphorylation of Smad2 protein. Then, we determined whether fucoidan also modulated the TGFβ3-induced non-Smad pathway. As shown in Fig. 4E and H, we found that fucoidan decreased the phosphorylation of ERK1/2 protein, which was induced by TGFβ3 treatment. Taken together, these data showed that fucoidan inhibition of cell viability in leiomyoma cells partly involves targeting TβR2, with the consequent attenuation of the β-catenin, Smad2 and ERK1/2 signaling pathways.

Inhibition of ULs growth and ECM accumulation in vivo by fucoidan

To investigate whether fucoidan can inhibit the growth of leiomyoma *in vivo*, we used ELT-3-LUC-xenografted mice as an experimental model. The treatment group received fucoidan (50 mg/kg) or vehicle (ddH_2O, control) via oral gavage every day for 42 days. During the

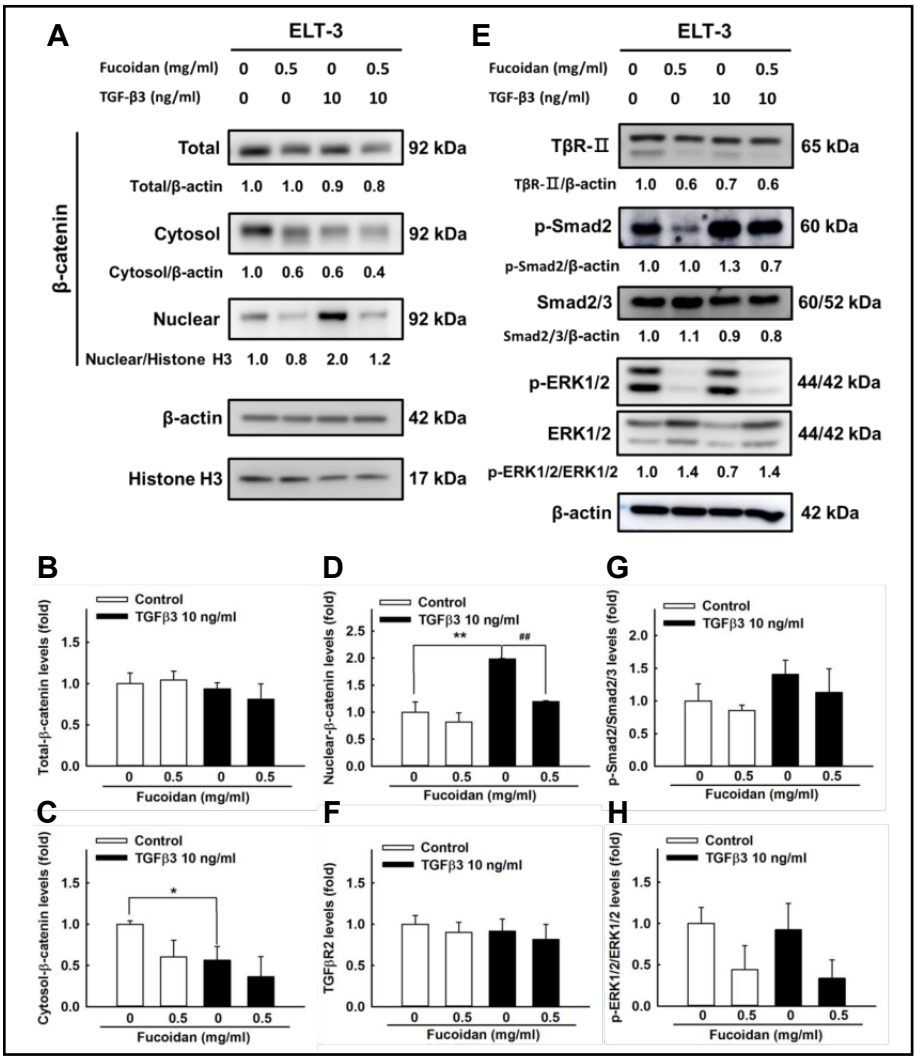

Fig. 4. Effect of fucoidan on TGFβR-mediated Smad or non-Smad signaling pathway in ELT-3 cells. ELT-3 cells were co-treated with 0.5 mg/ml fucoidan and TGFβ3 10 ng/ml for 48 h. Cell lysates were separated by SDS-PAGE and analyzed on western blots with the indicated antibodies. (A) Expression of β-catenin in the cytoplasm and nucleus. (E) Expression of Smad or non-Smad signaling pathway. β-actin was used as a loading control. The values of the band intensity represent the densitometric estimation of each band normalized by β-actin or Histone H3. Protein quantification of total-β-catenin (B), cytosol-β-catenin (C), nuclear-β-catenin (D), TβR-Ⅱ (F), p-Smad2 to Smad2/3 (G), and p-ERK1/2 to ERK1/2 (H) expression in ELT-3 cells was shown in bar graph. Significances were calculated with one-way ANOVA analysis and Tukey's HSD post-test. $^{*}p < 0.05$ and $^{**}p < 0.001$ compared with control; $^{\#\#}p < 0.001$ compared with TGFβ3 10 ng/ml.

treatment period, the body weights of the mice were measured each time they were gavaged to determine the possible side effects of fucoidan treatment on the overall health of the mice. IVIS in conjunction with a firefly bioluminescent enzyme was used to progressively track the growth of ELT-3-LUC tumors after fucoidan treatment in the mouse xenograft model (Fig. 5A). No significant difference in body weights of the mice was observed between the groups (Fig. 5B). Notably, a significant difference in tumor weight and volumes was observed between the control- and fucoidan-treated groups on day 42 of treatment (Fig. 5C and D). In addition, IVIS images showed an increase in bioluminescent signal strength in the control group, and in the quantitative chart (Fig. 5E), fucoidan-treated groups have significantly lower photon counts ($p<0.05$) compared with the control group. These data demonstrated the potent inhibitory effect of fucoidan on the growth of ULs within a relatively short treatment period.

To confirm the results of the animal study further, immunohistochemistry analysis was performed. Compared with the control group, the fucoidan-treated group showed a decrease in the proportion of PCNA (growth-promoting marker, Fig. 5H and I)-, fibronectin (ECM

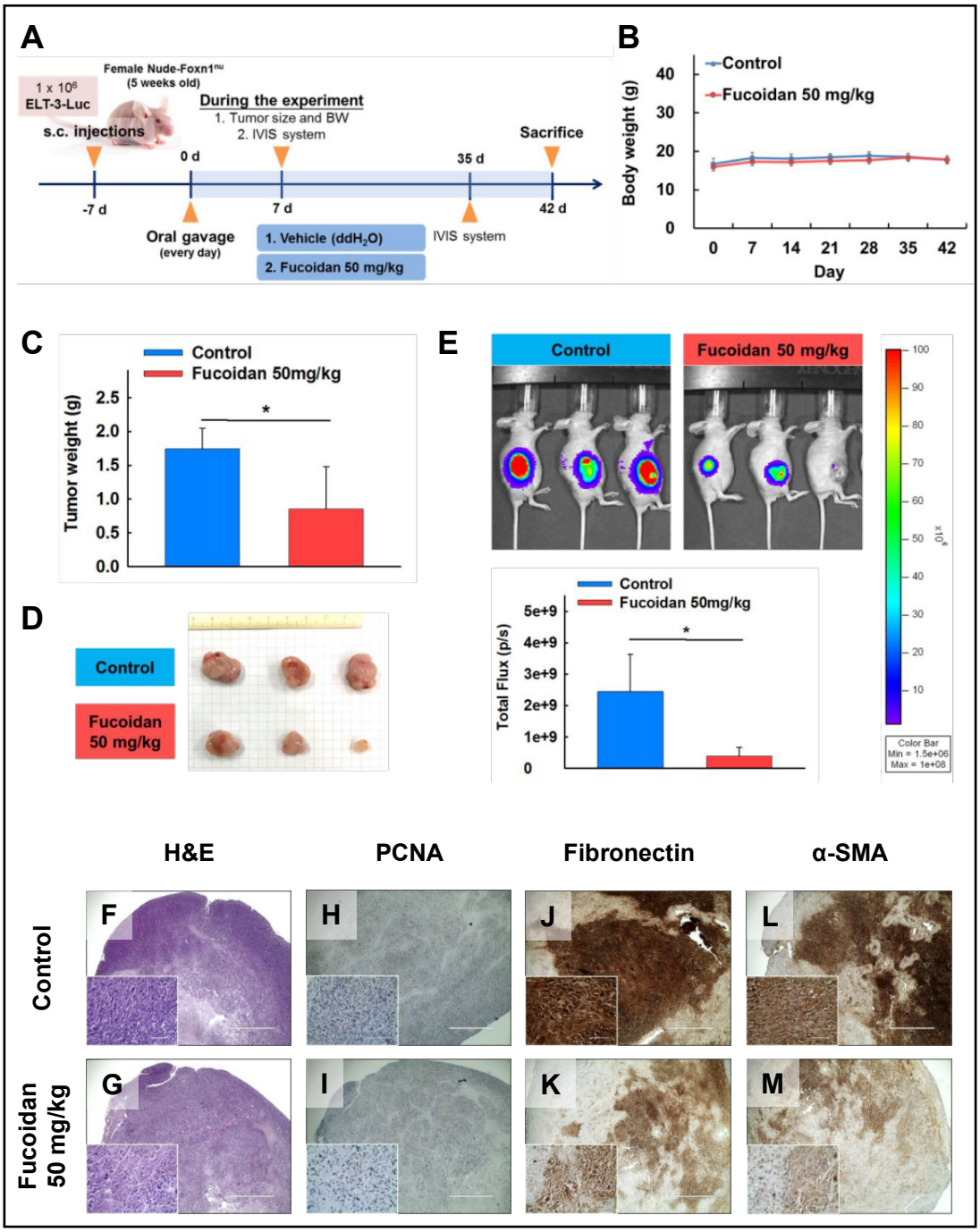

Fig. 5. *In vivo* effects of fucoidan using an ELT-3-LUC mouse xenograft model. (A) Schematic representation of treatment plan for ELT-3-LUC-xenografted mice. Tumors were implanted into the mice (n=3 per group) by subcutaneous injection of ELT-3-LUC cells (1 × 10^6 cells/100 µl PBS/Matrigel=1:1) on the right flank. After the tumors reached 50-100 mm^3 in size, the mice received every day with oral gavage of fucoidan (50 mg/kg) or vehicle (ddH_2O) for 42 days. (B) Body weight changes of the nude mice were measured. (C) and (D) Tumor weight and morphology of each group at day 42 after the treatment. (E) Total flux from luciferase imaging after heterotopic tumor cell injection. Significances were calculated with Student's t-test. *$p < 0.05$ compared with control. (F-M) Analysis of ELT-3-LUC tumor after fucoidan treatment. Tumors were excised and processed for hematoxylin and eosin (H&E) staining and immunohistochemical analysis of proliferating cell nuclear antigen (PCNA), fibronectin and alpha smooth muscle Actin (α-SMA). Original magnification, ×200.

marker, Fig. 5J and K)-, and α-SMA (myofibroblast marker, Fig. 5L and M)-positive cells, as well as hematoxylin and eosin staining (H&E stain, Fig. 5F and G). These data demonstrated that fucoidan suppress tumor progression and ECM accumulation in leiomyoma *in vivo*.

Discussion

In this study, we aimed to investigate the inhibitory effect of fucoidan on ULs growth both in ELT-3 cells and in a mouse xenograft model, and we found that fucoidan has potential benefits in inhibiting the growth of leiomyoma cells via decreasing ECM accumulation *in vitro* and *in vivo*.

In the inflammatory state, some factors, such as TGFβs, activin-A, TNF-α and estrogen, contribute to the differentiation into myofibroblasts during fibrosis. In general, myofibroblasts generate ECM to support tissue repair and homeostasis. However, during chronic inflammation, myofibroblasts are not limited by apoptosis and generate excessive contractions and ECM protein secretion (like collagen, fibronectin, etc.), leading to pathological fibrosis [8]. Current evidence suggests that α-SMA is a key component supporting tissue contraction of ECM [37]. Previous studies have demonstrated that α-SMA is elevated in leiomyoma compared to myometrium [38]. Yan et al. also found that the expression of α-SMA was significantly increased at 5 ng/ml TGFβ1 compared with the control in cardiac myocyte H9c2 cells [39]. In addition, fibronectin mRNA expression was higher in leiomyoma than in autologous myometrium, and the increase caused by TGFβ3 was markedly higher than the one caused by TGFβ1 [9]. Similarly, Levy et al. demonstrated that untreated leiomyoma showed a 2-fold increase in fibronectin protein concentration compared with untreated myometrial cells. Additionally, TGFβ3 treatment enhanced the expression of fibronectin in both myometrial and leiomyoma cells [40]. Earlier studies demonstrated that collagen type 1 (COL1A1) protein production was higher in untreated leiomyoma than myometrial cells, while Joseph et al. demonstrated that elevating TGFβ3 concentration of 0.1 ng/mL to 10 ng/mL resulted in concentration-dependent increase in COL1A1 in both myometrial and leiomyoma cells [41]. Our results from the histopathological analysis of the fucoidan-treated (50 mg/kg) group showed a lower expression of fibronectin and α-SMA on ELT-3-LUC-xenografted mice compared to the control group *in vivo*. In addition, *in vitro* experiment results also confirmed that fucoidan abrogates the TGFβ3-induced protein expression of fibronectin, α-SMA and COL1A1 in ELT-3 cells compared to controls.

The TGFβ superfamily comprises multifunctional peptides that have diverse biological activities, including regulating cell growth and differentiation and promoting ECM production [42]. The TGFβ subtypes consist of three isoforms (TGFβ1, TGFβ2 and TGFβ3) that have been found in mammals. The different TGFβ isoforms have their own receptors to regulate signaling pathways [43]. According to previous studies, the TGFβ3 isoform is the major inductor of elevated ECM production and decreased ECM degradation factor production in uterine leiomyoma [41]. Furthermore, uterine leiomyoma has 5-fold higher levels of TGFβ3 mRNA expression than autologous myometrium [11]. Therefore, we hypothesized that TGFβ3 would contribute to the dysregulated ECM in leiomyomas.

According to a previous study, the detection of the SP cell phenotype by Hoechst 33342 exclusion can identify cancer stem-like cells [36]. Human leiomyoma SP cells exhibit the potential ability of tumor-initiating cells, including a hypoxic cell culture proliferation ability, *in vitro* differentiation into adipocytes and osteocytes, and the capacity to give rise to leiomyoma-like tissue *in vivo* [44]. In our study, we also isolated putative leiomyoma stem cells from the ELT-3 leiomyoma cell fraction and found that these cells represented 2.0% of the total living cell population (fucoidan 0 mg/ml group, left panel). However, fucoidan treatment decreased the number of selected stem cells for SP distribution at 1 mg/ml, indicating that fucoidan can inhibit the activity of leiomyoma stem-like cells.

Cell growth and differentiation depend on the cell cycle. The cell cycle consists of four distinct phases: G1, S, G2 and M phase. In addition, cells can enter a state of quiescence called

G0 phase [45]. G1 progression and entry into S phase are controlled by cyclins D, which have been reported to be overexpressed in uterine leiomyoma compared with autologous myometrium [46]. According to the study from Cho et al, the treatment of bladder cancer cells with fucoidan induced G0/G1 phase arrest via a decreased expression level of cyclin D1/CDK4 and cyclin E/CDK2 [29]. Our results also demonstrated that fucoidan can induce sub-G1 phase arrest and down-regulated the protein expression of cyclin D1 in ELT-3 cells.

In general, sub G0/G1 cell accumulation suggests that dead cells/apoptotic cells are present. Several studies have also shown that fucoidan mediated cell death via triggering apoptosis. Treatment with 820 μg/mL of low molecular weight (LMW) fucoidan resulted in a significant decrease in anti-apoptotic proteins Bcl-2, Bcl-xl and Mcl-1 in MDA-MB231 breast cancer cells [35]. Similarly, Xue et al. also demonstrated that fucoidan treatment down-regulated the expression of Bid, Bcl-2 and Bcl-xl and up-regulated the level of Bax, especially at 25 μg/ml in MDA-MB231 breast cancer cells [47]. Our study had similar results, where fucoidan induced apoptosis via up-regulation of the Bax/Bcl-2 ratio in ELT-3 cells. As Bcl-2 family members are mainly involved in the intrinsic apoptosis pathway, we assume fucoidan can induce the intrinsic apoptosis pathway in our study.

The *in vivo* experiments verified our *in vitro* findings on the anti-proliferation and anti-fibrotic effects of fucoidan against leiomyoma. During the experiment for 42 days, we found that the fucoidan-treated (50 mg/kg) group had significantly reduced tumor volume in ELT-3-LUC xenograft mice compared with the control group. Similarly, Hsu et al. demonstrated that fucoidan-induced a marked dose-dependent reduction of tumor volume in male C57BL/6 mice xenografted with Lewis lung carcinoma (LLC1) cells, especially at a concentration of 96 mg/kg for 21 days, indicating that fucoidan exerted anti-tumor activity *in vivo* [48]. PCNA, which is a coenzyme of DNA polymerase that is involved in the synthesis of DNA in the nucleus, is commonly used as a marker of cell proliferation [49]. Our histopathological analysis showed that the proportion of PCNA-positive cells was decreased in the fucoidan-treated group at 42 days. Similar effects were reported in studies by Zhang et al, which demonstrated that EGCG treatment decreased the number of PCNA-positive cells at 4 and 8 weeks compared with water treatment (control) [14].

Many studies have demonstrated that TGFβ superfamily signaling is essential for female reproduction and that abnormal TGFβ signaling may promote reproductive diseases or cancers. TGFβ signals through a Smad-dependent (i.e., canonical) pathway to regulate the transcription of several target genes. In addition, intracellular signaling activated by TGFβ also includes Smad-independent (i.e., non-canonical) pathways, including various branches of MAP kinase pathways, Rho-like GTPase signaling pathways, and phosphatidylinositol-3-kinase/AKT pathways [42, 50].

ERK1/2 signaling can regulate proliferation, survival, and apoptosis in a variety of cell types. 17β-estradiol (E2) exposure rapidly activates ERK1/2 in leiomyoma cells but not in normal myometrium, indicating that aberrant ERK1/2 signaling occurs in leiomyoma [51]. In our study, we demonstrated that TGFβ3 binds to TβR2, leading to phosphorylated Smad2 and ERK1/2 level increases and ultimately gene transcription activation. However, fucoidan abrogates TGFβ3-induced protein expression of phosphorylated Smad2 and ERK1/2. Hsu et al. found similar effects in lung cancer cells, as TGFβ1 stimulation enhanced the phosphorylation of Smad2/3, Akt, and ERK1/2 but fucoidan abolished the TGFβ1-induced phosphorylation of these molecules [48]. In addition, several studies also investigated these signaling pathway with different treatment, such as Loureirin B (LB), He et al. found that LB inhibited the up-regulation of p-ERK that was induced by TGFβ1 in hypertrophic scar tissue [52].

Activated β-catenin is associated with proliferation and ECM formation in human leiomyoma tissue [53]. Ono et al. have demonstrated that the downregulation of WNT/β-catenin signaling, via inhibitor of β-catenin and TCF4 (ICAT), niclosamide, or XAV939, has antitumor effects on primary cultures of human leiomyoma cells *in vitro* [54]. TGFβ facilitates the translocation of un-phosphorylated β-catenin from the cytoplasm to the nucleus [55],

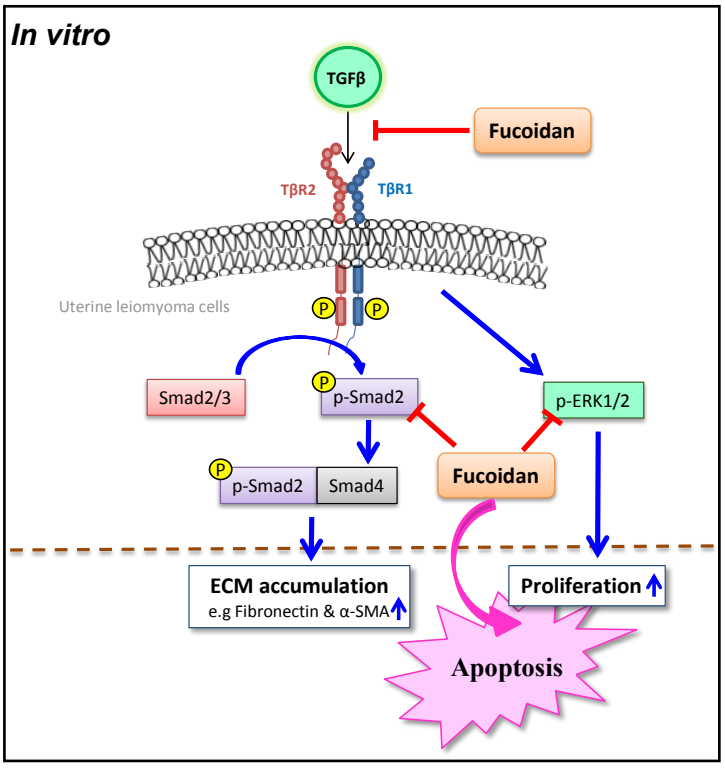

Fig. 6. Schematic diagram of the mechanism of fucoidan inhibits the growth of uterine leiomyoma. Fucoidan significantly abolished the cell viability of ELT-3 cells induced by TGFβ3, and promote sub-G1 phase arrest and apoptosis occur. Fucoidan also abrogated the ECM proteins up-regulated by TGFβ3 in ELT-3 cell via TGFβR-mediated Smad and ERK1/2 signaling pathway.

and via integration with the T-cell factor and lymphoid enhancer factor-1 (TCF/LEF1) family of transcription factors to activate WNT target gene expression [56]. In our study, we found that fucoidan treatment decreased the levels of β-catenin in both the cytoplasm and the nucleus in ELT-3 cells and abolished the TGFβ3-induced β-catenin translocation into the nucleus. Similarly, Xue et al. demonstrated that fucoidan treatment decreased the levels of β-catenin in the cytoplasm and nucleus of MDA-MB-231 cells [47].

Conclusion

The present study demonstrated that fucoidan significantly decreased the cell viability and stem cell activity of ELT-3 cells. In addition, fucoidan abolished the effect of TGFβ3 and induced sub-G1 phase arrest and apoptosis. Fucoidan also abrogated ECM protein up-regulation by TGFβ3 in ELT-3 cells via TGFβR-mediated Smad and ERK1/2 signaling pathways, providing a potential novel therapy in preventing hyperplasia of leiomyoma cells *in vitro* (Fig. 6). Furthermore, fucoidan suppresses tumor growth and decreased the expression of PCNA, fibronectin and α-SMA in tumor tissue *in vivo*. To the best of our knowledge, this is the first study to demonstrate the inhibitory potential of fucoidan on ULs growth *in vivo* and may encourage further studies to highlight the molecular mechanism involving fucoidan and ULs.

Abbreviations

ULs (Uterine leiomyoma); ELT-3 (Eker uterine leiomyoma cells); HUtSMC (Human uterine smooth muscle cells); ECM (Extracellular matrix); TGFβ (Transforming growth factor beta); PCNA (Proliferating cell nuclear antigen); α-SMA (Alpha-smooth muscle actin); SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis); IVIS (*In vivo* Imaging System); SP (Side population); CDKs (Cyclin-dependent kinase).

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SMH designed this research. HYC carried out most of the experiments, analyzed the data, drew the figures and drafted this manuscript. TCH and LCL helped with cell culture, western blot experiments and animal experiments. All authors read and approved the final manuscript.

Disclosure Statement

The authors declare that they have no competing interests.

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