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Targeting colon cancer stem cells with novel blood cholesterol drug pitavastatin

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Abstract. – OBJECTIVE: Previous studies have demonstrated the importance of stem cells in human cancer, including colon cancer. Pitavastatin is approved for the treatment of hyperlipidemia and has also been shown to inhibit stem cell proliferation in preliminary *in vitro* studies. This study was done to investigate the effects of pitavastatin on human colon carcinoma stem cells (coCSCs) *in vitro* and in mouse tumor xenografts *in vivo*.

MATERIALS AND METHODS: Human colon adenocarcinoma cell lines, SW480 and SW620, were cultured to the spheroid formation. The effects of pitavastatin on colon cancer stem cells were studied using the colorimetric MTT cell proliferation assay; quantitative polymerase chain reaction was used to determine the expression of cell cycle genes, *OCT4*, *SOX2*, and *NANOG*; Western blots were performed to measure MDR1. Mice were injected subcutaneously with SW480 cells; the growth of these tumor xenografts was studied using volumetric analysis following pitavastatin treatment.

RESULTS: Specific cell culture medium provided conditions that resulted in the expression of colon cancer stem cell markers when compared with normal cultured cells. Colon cancer stem cells were inhibited by pitavastatin treatment. Pitavastatin reduced the expression of stem cell markers of colon cancer stem cells and induced the cell apoptosis. Pitavastatin inhibited the growth of mouse tumor xenografts.

CONCLUSIONS: The findings of this preliminary study have demonstrated a potential role for pitavastatin in the inhibition of stem cell proliferation in colon carcinoma. Further studies are recommended to determine the mechanism of these effects on colon carcinoma cells *in vitro* and *in vivo*.

Key Words:
Pitavastatin, Colon cancer, Stem cells, Mouse xenograft, Cell proliferation.

Introduction

Colon cancer arises from the glandular epithelium and may be locally invasive or may metastasize to distant organs. In 2012, colorectal cancer was ranked as the third most common cancer in men and women worldwide, with an estimated 373,600 men and 320,300 women having died from this cancer in that year[1,2]. Colon cancer has a high mortality rate because it is difficult to detect and treat at an early stage. The current therapeutic methods for colon cancer are surgery, chemotherapy, and radiotherapy[3]. However, some colon cancers are resistant to traditional therapeutic approaches or may relapse following treatment[3]. All these factors mean that it is important to develop both improved detection and treatment approaches for colon cancer.

Recent studies[4,5] have shown that a small subpopulation of stem cells in human cancer, including colon cancer, may be associated with tumor growth, resistance to conventional therapy, and with patient prognosis. The theory that has developed is that cancer stem cells may provide a therapeutic target. There are now several surface proteins that are expressed by stem cells, including CD133, CD44, CD24, which are now considered to be markers for colon cancer stem cells[6-9]. There are also some recently identified proteins, including aldehyde dehydrogenase (ALDH), octamer-binding transcription factor 4 (OCT-4), SOX2, and the homeobox protein, NANOG, which are expressed by colon cancer stem cells[6-9]. There are now methods available to obtain cancer stem cells, including modified and serum-free culture systems[10,11]. Following isolation of cancer stem cells, different methods may be used to target them, or to screen them for their response to drugs[10,11].

Pitavastatin is a potent 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor, which has received approval by the US Food and Drug Administration (FDA) for the treatment of hypercholesterolemia[11]. In 2014, Jiang et al[12] reported that pitavastatin could inhibit human glioblastoma cells *in vitro*.

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The human colon cancer stem cell forms an important tumor subpopulation, remaining quiescent but expressing high levels of ATP-binding cassette (ABC) G2 transporters (ABCG2 or CDw338), and the Multi-Drug Resistance Gene, MDR1[13-15]. Because these stem cell transporter proteins may be able to export small therapeutic molecules out of cells, they may be involved in the development of drug resistance. Also, cancer stem cells are quiescent in the G0 phase of the cell cycle and are less likely to respond to chemotherapy or radiotherapy. Therefore, cancer stem cells may be a new approach to targeted therapy in oncology, including targeting the expression of the stem cell phenotype of cancer stem cells[16,17].

Recently, specific stem cell markers expressed by cancer stem cells have been studied, and because cancer stem cells can export some small molecules, oncolytic viruses have been shown to be a potential approach to the lysis of cancer stem cells[18-22]. However, studies on the effects of pitavastatin on colon carcinoma stem cells are still awaited. Therefore, the aim of this preliminary study was to investigate the effects of pitavastatin on human colon carcinoma stem cells *in vitro* and in mouse tumor xenografts *in vivo*.

Materials and Methods

Ethical Approval

This study was reviewed and approved by the local Ethics Committee and the Institutional Review Board (IRB) of Zhengzhou University, China.

Conduct of Animal Studies

All the animal studies were performed according to the guidelines U.K. Animals Act, 1986 and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Reagents

The chemotherapy drugs cisplatin and fluorouracil (5FU) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The drug pitavastatin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Cultures

The colon cancer cell lines, SW480 and SW620, were obtained from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. SW480 and SW620 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). The culture medium was added with penicillin and streptomycin.

Cell Spheroid Formation

The SW480 and SW620 cells were trypsinized to dissociate the cells, which were cultured in the untreated dishes. The cells were maintained in the DMEM: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Grand Island, NY, USA) without serum. The cell culture medium was supplemented with 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml basic fibroblast growth factor (bFGF). The cells were cultured for 7 days until spheroid formation occurred. These colon cancer stem cell spheroids were centrifuged, trypsinized, and used for further investigation.

Quantitative Reverse Transcription-polymerase Chain Reaction (qRT-PCR)

The colon cancer stem cells were lysed with TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was extracted following the protocol of TRIzol in isolating RNA. A 1 μg sample of RNA was used for reverse transcription using the SuperScript III Reverse Transcriptase kit (Thermo Fisher Scientific, Inc. Waltham, MA, USA) according to the manufacturer's protocol.

Primers Used

The primers for quantitative reverse transcription PCR are listed below.

OCT4 forward: AAGCGATCAAGCAGCGACTAT,
OCT4 reverse: GGAAAGGGACCGAGGAGTACA,
SOX2 forward: TTGCTGCCTCTTTAAGACTAGGA
SOX2 reverse: CTGGGGCTCAAACTTCTCTC
NANOG forward: CAAAGGCAAACAACCCACTT,
NANOG reverse: TCTGCTGGAGGCTGAGGTAT,
GAPDH forward: TCGGAGTCAACGGATTTGGT
GAPDH reverse: TTGCCATGGGTGGAATCATA

Cell Viability Measurements

The stem cell spheroids derived from SW480 cells and SW620 cells were trypsinized into single cells and split into 96-well dishes at 3,000 cells per well. The cells were treated with increasing doses of pitavastatin for 48 hrs or treated with 5 μM pitavastatin for increasing numbers of days. Cell viability was evaluated using the MTT assay (Beyotime Co., Jiangsu, China). Cells were incubated for 4 hrs, formazan was

dissolved in dimethyl sulfoxide (DMSO) (Beyotime Co., Jiangsu, China) and the absorbance was measured at a wavelength of 570 nm. The relative cell viability was calculated as the ratio of the indicated group with the control group without treatment.

Cell Proliferation Measurement

The colon cancer stem cells were split into 96 well dishes at 2,000 cells per well. Cell proliferation was evaluated using the MTT assay (Beyotime Co., Jiangsu, China) at indicated days. The absorbance was measured at the wavelength of 570 nm. The relative cell viability was calculated as the ratio of the indicated group with the control group (cells at day 0).

Cell Cycle Detection

The SW480 stem cells forming cell spheroids were treated with 5 μM pitavastatin for 48 hrs. The cells were collected, trypsinized into single cells, and fixed with 70% ethanol at 4°C for 30 min. The cells were washed three times with PBS and incubated with 50 μg/ml RNAase at 37°C for 30 min. The cells were then stained with 50 μg/ml propidium iodide (PI) for an additional 30 min in the dark at room temperature. Then, the cells were detected by a flow cytometry.

Western Blot for Protein Expression

The Western blot assay included primary antibodies to multidrug resistance (MDR1) protein and GAPDH (control) (Cell Signaling Technologies, Danvers, MA, USA). The secondary antibody goat anti-rabbit were used at dilutions of 1:3000 (Beyotime Co., Jiangsu, China). The Western blot assay was carried out based on the protocol from the Cell Signaling Technologies (Danvers, MA, USA).

Mouse Xenograft Studies

Four-week-old nude Balb/c mice were purchased from Shanghai Laboratory Animal Center (SLAC) (Shanghai, China), and raised for one week. The five-week-old mice were injected subcutaneously with 2 x 10^6 SW480 stem cells. The mice were given intraperitoneal injections of either saline or 10 mg/kg pitavastatin for 7 days. The tumor size was measured every day for each mouse in each treatment group (saline- or pitavastatin-treated). The tumor growth rate and mice survival curve were calculated at the end of the experiments.

Statistical Analysis

Comparative statistical analysis of treated and untreated cultured colon carcinoma cells and the treated and untreated mice were performed using Student's *t*-test. A p-value < 0.05 was considered to be statistically significant.

Results

Cultured Colon Cancer Stem Cell Spheroids

Colon cancer cell lines SW480 and SW620 formed cell spheroids by 7 days in the selective culture medium. Comparison between the cells from the cell spheroids and the confluent colon cancer cells in culture showed that the colon cancer stem cells were more resistant to chemotherapeutic drugs fluorouracil (5FU) and cisplatin (Figure 1A, 1B). The SW480 stem cells and SW620 stem cells showed increased expression of stem cell-associated genes OCT4, SOX2 and NANOG (Figure 1C). Also, the colon cancer stem cells were more quiescent, with a reduced cell proliferation rate (Figure 1D). These findings supported the view that the cell spheroids were colon cancer stem cells.

Pitavastatin Treatment and Colon Cancer Stem Cell Growth

When presumptive colon cancer stem cells from the cultured cell spheroids were treated with pitavastatin, cell growth was inhibited in a dose-dependent manner (Figure 2A). Pitavastatin treatment also reduced the expression of stem cell markers in the colon cancer stem cells (Figure 2B).

Pitavastatin Treatment and Colon Cancer Stem Cell Apoptosis

Comparison of the non-treated or pitavastatin-treated stem cells derived from the cultured colon carcinoma cell spheroids showed that pitavastatin increased the sub-G1 population of the stem cells (Figure 3A, 3B). These findings indicated that pitavastatin induced stem cell apoptosis. Furthermore, pitavastatin treatment was associated with a decrease in expression levels of MDR1 protein, an important ATP-binding cassette (ABC) transporter in cancer stem cells (Figure 3C).

Pitavastatin Treatment and the Growth of Colon Cancer Stem Cell Xenografts

The tumor xenografts in the mice treated with pitavastatin grew more slowly when compared

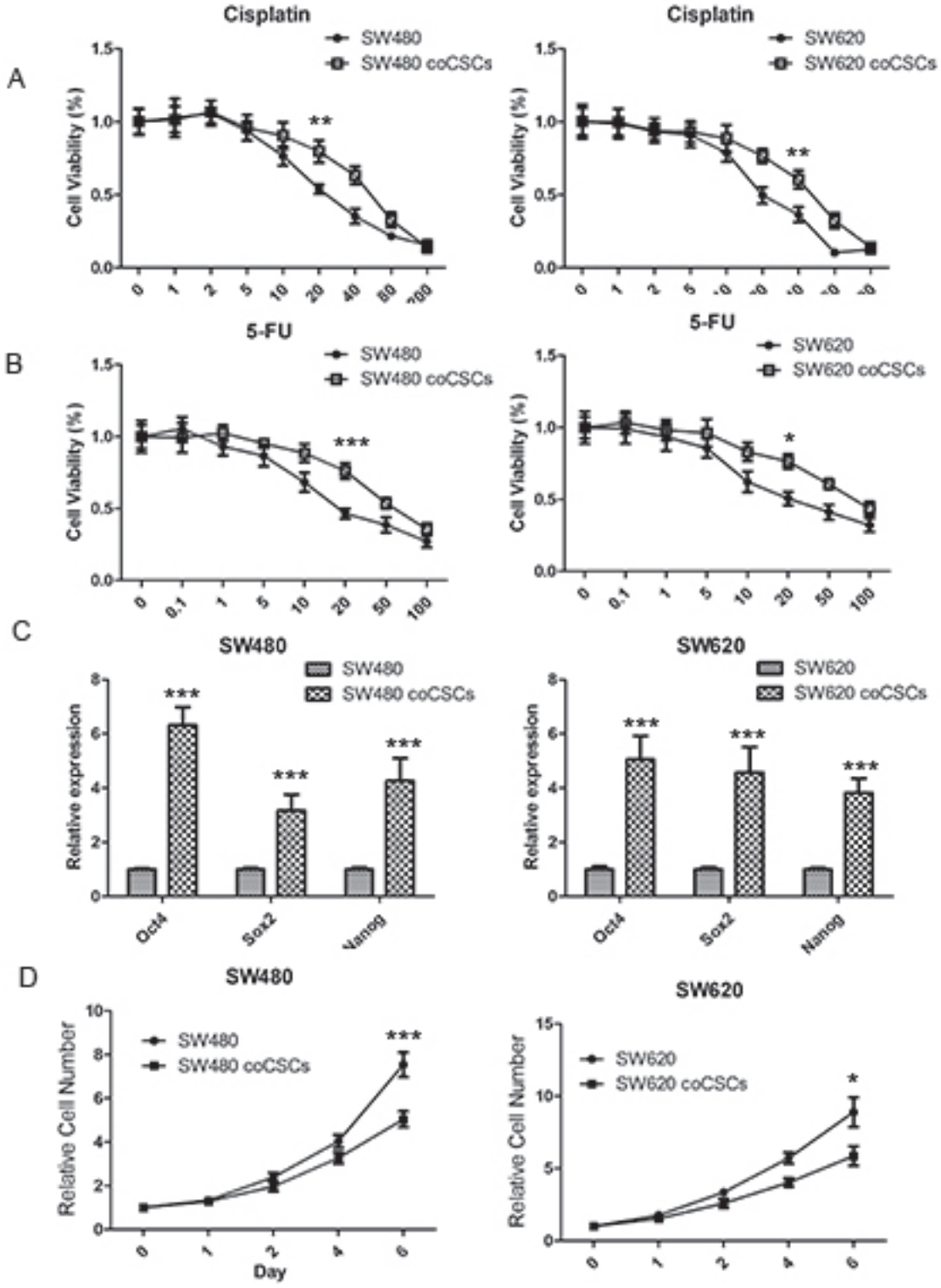

Figure 1. Colon cancer cell spheroids are colon cancer stem cells. ***A,*** Treatment of SW480 colon cancer cells and SW480 colon cancer stem cells or SW620 colon cancer cells and SW620 colon cancer stem cells with cisplatin at indicated doses. ***B,*** Treatment of SW480 colon cancer cells and SW480 colon cancer stem cells or SW620 colon cancer cells and SW620 colon cancer stem cells with fluorouracil (5-FU) at indicated doses. ***C,*** Stem cell genes expression in SW480 colon cancer cells and SW480 colon cancer stem cells or SW620 colon cancer cells and SW620 colon cancer stem cells. D, Proliferation rate of SW480 colon cancer cells and SW480 colon cancer stem cells or SW620 colon cancer cells and SW620 colon cancer stem cells. * mean p <0.05, ** mean p <0.01, *** mean p <0.001.

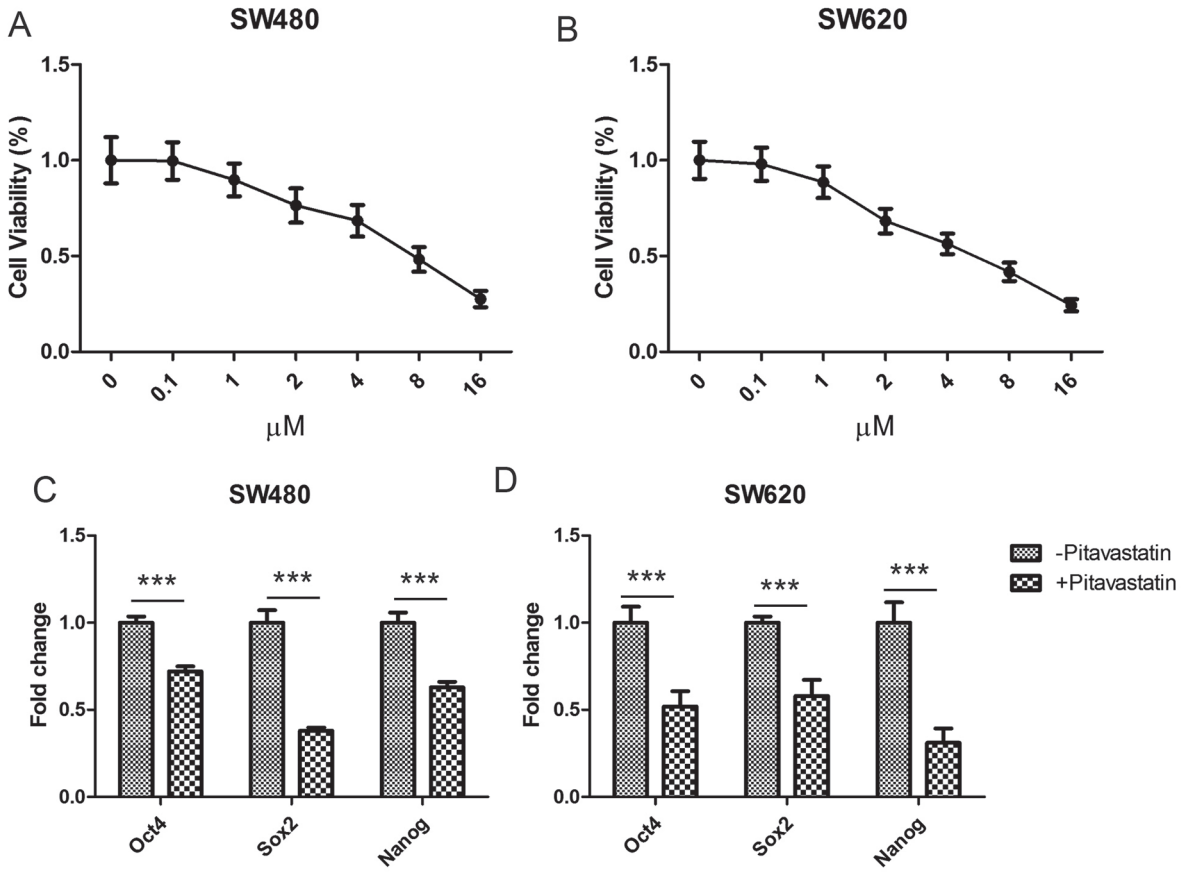

Figure 2. Pitavastatin decreased colon cancer stem cells growth. ***A,*** Cell viability detection of SW480 colon cancer stem cells with or without pitavastatin treatment. ***B,*** Cell viability detection of SW620 colon cancer stem cells with or without pitavastatin treatment. ***C,*** Stem cell gene expression in SW480 colon cancer stem cells with or without pitavastatin treatment. ***D,*** Stem cell gene expression in SW620 colon cancer stem cells with or without pitavastatin treatment. *** mean $p < 0.001$.

with the control group mice treated with saline injection (Figure 4A). Survival study data showed that pitavastatin significantly promoted the survival of mice with colon cancer tumor xenografts (Figure 4B).

Discussion

Worldwide, colon cancer is one of the leading causes of cancer-related death[1, 2]. The development of effective drug treatments for colon cancer is of increasing importance, particularly given the increase in tumor drug resistance. By making malignant tumors more sensitive to chemotherapy or radiotherapy, an approach to more effective cancer therapy may be to target cancer stem cells, including colon cancer stem cells[10,11,16]. The lipid-lowering drug, pitavastatin, has previously been reported to inhibit the proliferation of human glioblastoma cells *in vitro*[12], but this preliminary study is, we believe, the first to demonstrate the effects of pitavastatin on human colon carcinoma cells.

In this study, we generated colon cancer stem cells from the colon cancer cell lines, SW480 and SW620. The putative stem cells were then compared with the non-spheroidal cells in culture. Comparison between the putative colon cancer stem cells and the remaining cells in culture showed increased resistance of the former to the chemotherapy drugs fluorouracil (5-FU) and cisplatin. The colon cancer stem cells grew more slowly, indicating a more quiescent state. Also, the stem cells showed increased gene expression of OCT4, SOX2, and NANOG. These findings supported the stem cell phenotype of the colon cancer cell spheroids studied *in vitro*.

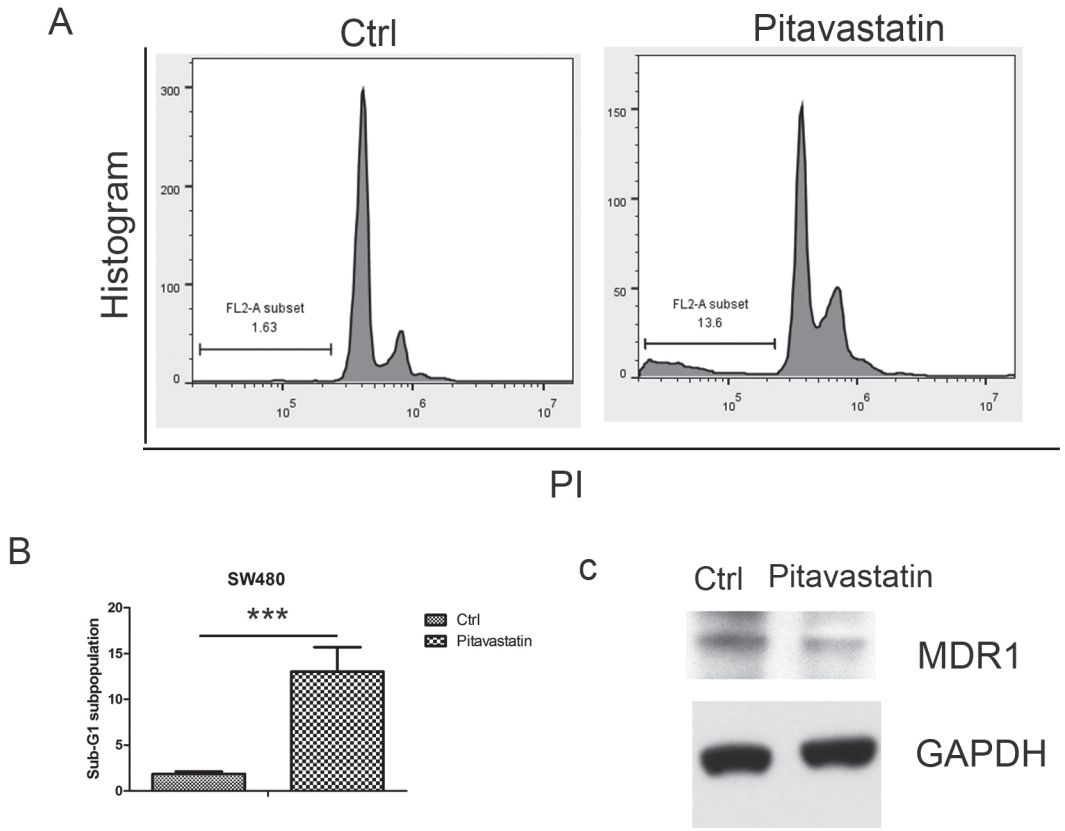

Figure 3. Pitavastatin induced colon cancer stem cells apoptosis. ***A,*** Cell cycle detection of the sub-G1 population in SW480 colon cancer stem cells with or without pitavastatin treatment. ***B,*** Quantitative analysis of sub-G1 subpopulation. ***C,*** Detection of MDR1 expression after pitavastatin treatment. GAPDH was used as an internal control. *** mean $p < 0.001$.

When the effects of pitavastatin were examined on the colon cancer stem cells, pitavastatin inhibited stem cell growth and induced stem cell death, or apoptosis. The *in vivo* tumor xenograft studies in mice further demonstrated the efficiency of pitavastatin in targeting colon cancer stem cells. We also found that pitavastatin could reduce the expression of MDR1, an ATP-binding cassette (ABC) transporter protein. Pitavastatin is a small molecule that may induce colon cancer stem cell death due to its ability to reduce the expression of MDR1 protein, leading to the inability of colon cancer stem cells to export small molecules. The findings of this study indicate that pitavastatin may have a future role in combination with chemotherapy drugs for colon cancer by its tar-

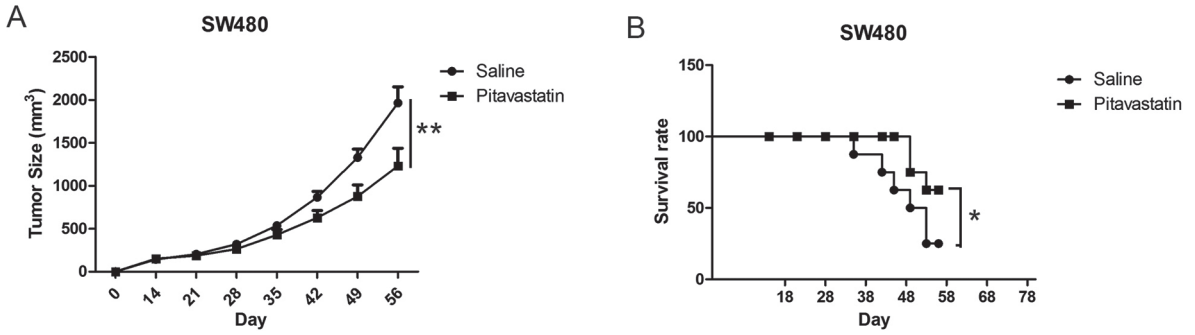

Figure 4. Pitavastatin inhibited colon cancer stem cells *in vivo*. ***A,*** Tumor growth rate measurement with or without pitavastatin treatment. ***B,*** Mice survival curves with or without pitavastatin treatment. * mean $p < 0.05$, ** mean $p < 0.01$.

geting effects on colon cancer stem cells. Besides the novel function of pitavastatin, there are also reports about another novel drug for cancer stem cell targeting, like thioridazine, salinomycin, which have been screened and applied in targeting various types of cancer stem cells[23, 24]. If we can develop these small molecules as well as other methods for cancer stem cells targeting, the cancer therapy will be more efficient[25].

This study was preliminary in nature and had several limitations. Two colon cancer cell lines, SW480 and SW620, were chosen, without the inclusion of a normal colonic epithelial cell line. A limited panel of cancer stem cell markers was used, which means that the stem cell nature of the cells in the tumor spheroids was presumptive. For future studies, we will isolate the cancer stem cells from patients tissue. The efficiency of pitavastatin in targeting colon cancer stem cells may be further improved by combination with oncolytic viruses. Xu et al[19] disclosed in their study that oncolytic adenovirus with novel therapeutic gene acetylcholinesterase dramatically inhibited gastric cancer stem cell proliferation. The combination of pitavastatin with oncolytic viruses may get enhanced ability in targeting colon cancer stem cells. Other small molecules, like thioridazine, salinomycin[23,24], can also be used in combination with pitavastatin to get high efficiency in targeting colon cancer stem cells.

Conclusions

The findings of this preliminary study have demonstrated a potential role for pitavastatin in the inhibition of stem cell proliferation in colon carcinoma *in vitro* and *in vivo*. Further studies are recommended to determine the mechanism of these effects on colon carcinoma cells in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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