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Das, Swadesh K.  
Hashimoto, Takashi  
Kanazawa, Kazuki

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**Growth inhibition of human hepatic carcinoma HepG2 cells by fucoxanthin is associated with down-regulation of cyclin D**

Swadesh K. Das<sup>1</sup>, Takashi Hashimoto<sup>1</sup> and Kazuki Kanazawa<sup>1\*</sup>.

<sup>1</sup>Laboratory of Food and Nutritional Chemistry, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai, Kobe, Nada, Hyogo 657-8501, Japan.

**\*Request for reprints:** Kazuki Kanazawa, Laboratory of Food and Nutritional Chemistry, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai, Kobe, Hyogo 657-8501, Japan. Tel. & Fax: +81-78-803-5879.  
E-mail: kazuki@kobe-u.ac.jp

**Running title:** Fucoxanthin induces growth arrest in HepG2 cells.

**Keywords:** fucoxanthin; cyclin D; cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> phase; proteasome; hepatocarcinoma cells.

## **Abstract**

Fucoxanthin, a major carotenoid in brown sea algae, has recently been demonstrated by us to inhibit the proliferation of colon cancer cells, and this effect was associated with growth arrest. These results, taken together with previous studies with fucoxanthin, suggest that it may be useful in chemoprevention of other human malignancies. The present study was designed to evaluate the molecular mechanisms of fucoxanthin against hepatic cancer using the human hepatocarcinoma HepG2 cell line (HepG2). Fucoxanthin reduced the viability of HepG2 cells accompanied with the induction of cell cycle arrest during the G<sub>0</sub>/G<sub>1</sub> phase at 25  $\mu$ M. This concentration of fucoxanthin inhibited the phosphorylation of the retinoblastoma protein (Rb) at Serine 780 (Ser780) position 18 h after treatment. The kinase activity of cyclin D and cdk4 complex, responsible for the phosphorylation of Rb Ser780 site, was down-regulated 18 h after the treatment. Western blotting analysis revealed that the expression of cyclin D-type protein was suppressed by treatment of fucoxanthin. This reduction was partially blocked by concurrent treatment with the proteasome inhibitor MG132, indicating the involvement of the proteasome-mediated degradation. In addition, RT-PCR analysis revealed that fucoxanthin also appeared to repress cyclin D mRNA. Thus, both the protein degradation and transcriptional repression seems to be responsible for suppressed cyclin D level in fucoxanthin-treated HepG2 cells which may be related to the antitumorigenic activity.

## 1. Introduction

Hepatocellular carcinoma (HCC) is the fifth leading cause of cancer mortality and the third most common cause of cancer-related death [1]. Most of the cases of hepatic cancer are not curable due to extensive liver dysfunction caused by concomitant cirrhosis, infrequent diagnosis at an early stage, and lack of appropriate chemotherapy. However, a number of epidemiological studies demonstrated that the risk of liver cancer might be reduced by the consumption of vegetables [2-4]. Among the various components of vegetables, carotenoids are one of the major active compounds, which can prevent different types of human malignancies like colon [5, 6], lung [7], and breast [8] cancer. Regarding HCC, protective role of carotenoids have also been reported. In a cohort study, lower plasma levels of  $\beta$ -carotene, the most widely studied carotenoid, appeared to be more predictive of elevated HCC risk associated with smoking and alcohol-drinking groups [9]. In addition, treatment with  $\beta$ -carotene has been shown to reduce the incidence of diethylnitrosamine-initiated and phenobarbital-promoted hepatocarcinogenesis in an animal model [10]. Other carotenoids like canthaxanthin, astaxanthin, lutein, and lycopene are also capable of suppressing carcinogen-induced HCC in animal models [11-13].

The growth of cells is normally determined by extracellular signals that control the gene expression and protein regulation required for cell division [14]. In contrast, during tumor progression, cancer cells are conferred with the capacity to proliferate independently of exogenous growth-promoting or growth-inhibitory signals [15, 16]. Thus, the antiproliferative effect of chemicals or drugs on cancer cells is one of the mechanistic ways to exert their anticarcinogenic activity. A number of compounds have shown their antineoplastic effects on HCC by inducing cell cycle arrest or apoptosis.

For example, acyclic retinoid, a derivative of retinoid used for several clinical trials [17, 18], inhibits proliferation by inducing cell cycle arrest in hepatic carcinoma cells [19]. Cisplatin and methoxymorpholinyl doxorubicin, clinically used as chemotherapeutic drugs against several cancers, proved preventive effect against HCC by inhibiting cellular proliferation [20, 21].

Fucoxanthin, whose structure is shown in Fig. 1, is an oxygenated carotenoid available in different types of edible seaweed such as *Laminaria japonica*, *Undaria pinnatifida*, and *Hijikia fusiformis*. Many biological functions of this compound have been studied; e.g., suppressive effect on adipocyte differentiation [22], antimutagenicity [23], anti-ocular inflammation [24], and cancer preventing effects. In our previous study [25], fucoxanthin exerted an antiproliferative effect by inducing cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> phase in human colon carcinoma cells. The oral administration of fucoxanthin suppressed the development of aberrant crypt foci, a pre-neoplastic marker for colon neoplasia, in azoxymethane-treated mice [26], and also showed chemopreventive effects against *N*-ethyl-*N*'-nitro-*N*- nitrosoguanidine-induced mouse duodenal carcinogenesis [27], two-stage mouse skin carcinogenesis [23], and 1,2-dimethylhydrazine-induced colon carcinogenesis [28]. Concomitantly, this compound has been shown to have growth inhibitory effects on various cell lines; e.g., prostate cancer PC-3, DU 145, and LNCaP cells [29], leukemia HL-60 cells [30], colon cancer HT-29, Caco-2, and DLD-1 cells [31], and neuroblastoma GOTO cells [32]. As hepatocarcinogenesis is concerned, fucoxanthin suppressed the spontaneous tumorigenesis in the liver of C3H/He male mice [23]. However, the mechanism by which fucoxanthin exerts these anticarcinogenic effects is unknown.

In the present study, hepatocarcinoma HepG2 cells were employed to elucidate the preventative mechanism of fucoxanthin against HCC.

## **2. Materials and methods**

### *2.1. Materials*

Propidium iodide (PI) and RNase A were purchased from Sigma Chemical (St. Louis, MO). For cell culture, Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan), and fetal bovine serum (FBS) was from Sigma Chemical. A proteasome inhibitor, MG132, and a fluorogenic peptide substrate, succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methyl-coumaryl-7-amide (Suc-Leu-Leu-Val-Tyr-MCA), were purchased from Peptide Institute, Osaka, Japan. All other reagents were of the highest grade available from commercial sources.

### *2.2. Preparation of fucoxanthin*

Fucoxanthin used in this study was isolated from the brown sea algae *Laminaria japonica* (kombu). Raw kombu was extracted with methanol and treated with charcoal to remove chlorophylls. The extract was concentrated in a rotary evaporator and subjected to preparative HPLC for the purification of fucoxanthin. The purity of the fucoxanthin used in this study was >95% as determined by HPLC. Fucoxanthin was dissolved in tetrahydrofuran (THF) at 20 mM as the stock solution.

### *2.3. Cell culture*

The human hepatocarcinoma HepG2 cells (a kind gift from Dr. Koji Ikura, Department of Applied Biology, Faculty of Textile Science, Kyoto Institute of Technology, Kyoto, Japan) were maintained with DMEM containing 10% heat-inactivated FBS, 4 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml

streptomycin in a humidified atmosphere of 95% air-5% CO<sub>2</sub> at 37°C. For all experiments, cells were seeded at a density of  $1 \times 10^5$  cells/ml, and cultured with media containing fucoxanthin or 0.5% THF following overnight pre-culture.

#### *2.4. Determination of cell viability*

Cell viability was determined by MTS assay according to manufacturer's protocol (Promega Corporation, Madison, WI). In brief, cells were seeded onto 96-well multiplates and treated with different concentrations of fucoxanthin or vehicle alone. Cells were added to MTS reagents [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], and incubated for 2 h at 37°C. The resulting MTS-formazan was dissolved in the culture media by agitation, and determined by the absorbance at 492 and 630 nm (for background) with a multiplate reader.

#### *2.5. Cell cycle analysis*

Cell cycle was analyzed by a published method [33] with slight modifications. Briefly, cells cultured on 60-mm dishes were harvested by trypsinization, washed with ice-cold PBS, and fixed with ice-cold 70% ethanol for at least 2 h at -20°C. The fixed cells were washed with PBS, incubated with 1 mg/ml RNase A and 50 µg/ml PI in PBS at room temperature for 30 min in the dark, and subjected to a flow cytometer (EPICS-XL, Beckman Coulter, Fullerton, CA) equipped with a EXPO32 flow cytometry software (Beckman Coulter). The data as analyzed by using a Multicycle software (Phoenix Flow Systems, San Diego, CA).

#### *2.6. Western Blotting Analysis*

Cells cultured on 100-mm dishes were washed with PBS and scrapped with RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Nonidet P-40, 1% sodium

deoxycholic acid, and 0.05% SDS). The protein concentration in the cell lysate was measured by the Lowry method [34]. Aliquots of 20 µg were subjected to SDS-PAGE and transferred onto poly-vinylidene difluoride membranes (Amersham Bioscience, Piscataway, NJ). Immunoblotting was performed according to the manufacturer's protocols with regard to the primary antibodies, with slight modifications. The sources of primary antibodies used in this study were as follows: Cell Signaling Technology (Beverly, MA), phospho-Rb (Ser780), Rb (4H1), p21<sup>Waf/Cip1</sup>, p57 and p16; Santa Cruz Biotechnology (Santa Cruz, CA), cyclin D1 and D3, cdk4, and β-actin; and Dako (Kyoto, Japan), p27<sup>Kip1</sup>. As secondary antibodies, horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG antibodies were purchased from Amersham Bioscience and Nacalai Tesque (Kyoto, Japan), respectively. The immunocomplexes were detected by enhanced chemiluminescence using ECL Plus Western Blotting System (Amersham Bioscience).

#### *2.7. Immunoprecipitation kinase assay*

The cyclin D/cdk4 kinase assay was performed as described previously [35, 36] with modifications. Briefly, cells cultured on 100-mm dish were treated with 25 µM fucoxanthin or vehicle as a control. Eighteen hours after the treatment, cells were washed twice with ice-cold PBS and harvested with a kinase buffer (25 mM Tris-HCl, pH 7.5 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM MgCl<sub>2</sub>, and 1 mM phenyl-methylsulfonyl fluoride (PMSF)). After measuring the protein content by the Lowery method [34], cdk4 was immunoprecipitated from the cell extract (400 µg of protein) using an anti-cdk4 monoclonal antibody (Santa Cruz Biotechnology) diluted 1:100 overnight. The immunocomplexes were added to 20 µl of protein A/G PLUS-Agarose (Santa Cruz Biotechnology), incubated at 4°C for 2 h, and washed at 4°C four



times with ice-cold kinase buffer. Immunoprecipitated cdk4 was incubated with 1 µg Rb-C fusion protein (Cell Signaling Technology) and 200 µM ATP in kinase buffer at 30°C for 30 min. Phosphorylation of Rb-C fusion protein was detected by Western blotting with antiphospho-Rb (Ser780) polyclonal antibodies.

## 2.8. *In vitro* proteasome activity assay

An *in vitro* proteasome activity assay was performed as described previously [37] with slight modifications. Briefly, HepG2 cells cultured on 100-mm dish were treated with vehicle or fucoxanthin (25 µM) for 12 h and then scrapped with RIPA buffer. After measuring protein concentration by the Lowry method, the cell lysate containing 10 µg of protein was immediately incubated at 30°C for 30 min with 60 µM of synthetic fluorogenic substrate, Suc-Leu-Leu-Val-Tyr-MCA, in 1ml of 20 mM Tris-HCl pH 8.0 containing 1 mM ATP and 2 mM MgCl<sub>2</sub>, and then 1 ml cold ethanol was added as a reaction stop solution. 7-Amino-4-methylcoumarin (AMC) liberated from the substrate by proteolytic hydrolysis was measured fluormetrically at 380 nm excitation and 440 nm emission.

## 2.9. Reverse transcriptase-polymerase chain reaction (RT-PCR) assay

Total RNA was isolated from HepG2 cells using Sepasol-RNA I super (Nacalai Tesque) according to the manufacturer's protocol. Aliquots (2 µg) of total RNA was reverse-transcribed into cDNA and amplified by using Ready to Go RT-PCR beads (Amersham Bioscience) in a thermal cycler(Bio-Rad, CA) with corresponding primers: Cyclin D1 [19], 5'-GTCACACTTGATCACTCTCC -3' (reverse) and 5'-CTGGCCATGAACTACCTGGA-3' (forward); cyclin D3 [38], 5'-CTGGCCATGAACTACCTGGA-3'(forward) and 5'-CCAGGAAATCATGTGCAATC-3'(reverse); and β-actin [19], 5'-

CCAGGCACCAGGGCGTGATG-3' (forward) and 5'-CGGCCAGCCAGGTCCAGACG-3' (reverse). PCR was conducted for 32 cycles and each cycle consisted of denaturation (40 sec at 92°C), annealing (40 sec at 58°C), and extension (50 sec at 72°C). Ten microliters of the reaction products were electrophoresed in 1.8% agarose gels, and cDNA fragments (Cyclin D1, 483 bp; Cyclin D3, 247 bp; and  $\beta$ -actin, 436 bp) were stained with ethidium bromide, and visualized by UV irradiation.

### *2.10. Statistical analysis*

The data are reported as the mean  $\pm$  SD of the values from three independent determinations. Statistical analysis was performed using Student's *t* test. Probability values less than 0.05 were considered statistically significant.

## **3. Results**

### *3.1. Fucoxanthin inhibits the growth of human hepatoma cells through cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> phase*

The effects of fucoxanthin on cell viability in HepG2 cells were investigated by an MTS assay (Fig. 2A). Fucoxanthin dose-dependently suppressed cell growth at concentrations of 10, 25 and 50  $\mu$ M 72 h after treatment, the end time point of this study compared with vehicle alone (0.5% THF). However, at 24 h, only 25 and 50  $\mu$ M fucoxanthin showed a significant growth inhibition. In a cell population, cell growth is the process deriving from the balance of cell proliferation and cell death. Then, the effect of fucoxanthin on the cell proliferation was investigated by flow cytometry in PI-stained cells at 24 h and earlier time points (Fig 2B). Fucoxanthin caused a significant accumulation of cells in G<sub>0</sub>/G<sub>1</sub> phase compared with only vehicle treated cells (48.9 $\pm$

2.8 vs.  $37.9 \pm 0.9$ ) within 18 h. At this time point we did not find any sub-G1 phase, an index of the apoptotic DNA fragmentation (data not shown). Thus, the inhibition of growth by 25  $\mu$ M of fucoxanthin resulted from a cell cycle arrest.

### *3.2. Effects of fucoxanthin on phosphorylation of Rb protein*

G<sub>0</sub>/G<sub>1</sub> arrest has been implicated in the hypo-phosphorylation of the retinoblastoma (Rb) protein [39]. Western blotting analysis was performed for the serine phosphorylation site (Ser780) of the Rb protein (Fig. 3A) to elucidate the effect of fucoxanthin on the phosphorylation level of Rb. Fucoxanthin at 25  $\mu$ M reduced the level of phosphorylated Rb at 12~18 h and at 24 h the effect was more apparent, while the level of total Rb protein remained constant. Because the Ser780 position of Rb protein is phosphorylated by the cyclin D and cdk4 complex (cyclin D/cdk4) [39], the effect of fucoxanthin on the kinase activity of the complex was investigated.

Fucoxanthin inhibited the activity of cyclin D/cdk4 in HepG2 cells 18 h after treatment (Fig. 3B). Thus, the decrease in the phosphorylation level of Rb protein appeared to be mediated by the suppression of cyclin D/cdk4 activity.

### *3.3. Fucoxanthin caused down-regulation of D-type cyclin*

The activity of cyclin and cdk complex is inhibited by several mechanisms, e.g., degradation of cyclin or inducing cdk inhibitor proteins [40]. The effects of fucoxanthin on the protein levels of D-type cyclins (cyclin D1 and D3), cdk4, and p27<sup>Kip1</sup> were examined. Fucoxanthin at 25  $\mu$ M decreased cyclin D1 and D3 levels as early as 12 h following treatment, and the effect was more evident at 18 h or 24 h time point. On the other hand, the protein level of cdk4 was unchanged at 12 h (Fig. 4A). The protein level of p27<sup>Kip1</sup> did not vary 24 h after treatment though it increased at 36 h (Fig. 4B). The expression of other kinase inhibitor proteins like p21<sup>Waf/Cip1</sup>, p53, and p16 was not

altered by treatment with fucoxanthin until 48 h after the treatment (data not shown).

These results indicate that the inhibitory effect of fucoxanthin on the activity of cyclin D/cdk4 is due to the decreasing levels of cyclin Ds.

#### *3.4. Fucoxanthin induces proteolysis of both cyclin D1 and cyclin D3*

Since cyclins tagged with ubiquitin are usually recognized and degraded by the 26S proteasome [41], the proteasomal activity was measured in fucoxanthin-treated HepG2 cells by using a fluorogenic substrate. Fucoxanthin significantly increased the proteasomal activity in HepG2 cells 12 h after treatment compared with the control cells (Fig. 5A). To investigate whether the level of cyclin D was regulated by proteosomal degradation in fucoxanthin-treated HepG2 cells, cells were pre-cultured with proteasome inhibitor MG132 for 30 min and then treated with 25  $\mu$ M fucoxanthin. Western blotting analysis demonstrated that MG132 inhibited the fucoxanthin-induced decrease in cyclin D1 and D3 (Fig. 5B), indicating that the decrease in these cyclins is at least partially mediated by proteosomal degradation. Thus, fucoxanthin up-regulated proteosomal degradation and decreased the protein levels of cyclin D1 and D3.

#### *3.5. Fucoxanthin causes a decrease in the cellular levels of cyclin D1 and cyclin D3 at the mRNA level*

In MG132-pretreated cells, the treatment with fucoxanthin slightly decreased protein levels of cyclin D1 and D3 compared with the protein levels in fucoxanthin-untreated cells (Fig. 5b). Therefore, the effect of fucoxanthin on the expression of the cyclin D mRNA was examined by an RT-PCR assay. The treatment with 25  $\mu$ M fucoxanthin significantly decreased the mRNA levels of cyclin D1 at 12 h and totally disappeared at 18 h (Fig. 6). A slight change of cyclin D3 mRNA level was observed at 12 h following treatment of fucoxanthin. Thus, fucoxanthin induced both proteolysis

and transcriptional suppression of cyclin Ds, resulting in the down-regulation of cyclin Ds.

#### **4. Discussion**

The present results characterized the growth inhibitory effect of fucoxanthin on the human hepatic cancer cell line. The effect was primarily due to an arrest in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle and apoptosis was not observed under the present condition. It is likely that the fucoxanthin possesses cytostatic rather than cytotoxic activity in these cells and then we further investigated the mechanisms of cell-cycle regulation. The phosphorylation of Rb plays a crucial role in the progression of G<sub>1</sub> phase and the transition of G<sub>1</sub> to S phase [42]. A reduction in the phosphorylation of Rb protein at Ser780 was detected. Ser780 in pRb is a specific site for phosphorylation by the cyclin D-cdk 4 complex in the G<sub>0</sub>/G<sub>1</sub> phase [39]. As this fact, treatment with fucoxanthin resulted in an inhibition of cyclin D/cdk4 activity in HepG2 cells.

Cdks are regulated by a distinct protein series; by cyclins required for the cdk activity and cdk inhibitor (CKIs) proteins [40]. Western blot analyses demonstrated that fucoxanthin decreased cyclin D1 and D3 protein levels. On the other hand, the protein levels of cdk4 and CKIs like p27<sup>Kip1</sup>, p21<sup>Waf1/Cip1</sup>, p57, and p16 are not affected by fucoxanthin, at least, not within a 12 h-treatment period. Therefore, the inhibitory effect of fucoxanthin on activity of the cyclin D/cdk4 is due to decreasing protein levels of cyclin Ds rather than due to CKIs.

D-type cyclin is unstable with a short half-life and degraded mainly via the 26S proteasome in an ubiquitin-dependent manner [41, 43]. Ubiquitin is a 76-amino acid polypeptide highly conserved in eukaryotic cells. Subsequent attachment of ubiquitin

monomers to the substrates results in multi-ubiquitinated chains, followed by degradation through the 26S proteasome [44]. A number of therapeutic agents have been observed to induce cyclin D degradation *in vitro* through proteasome degradation [45-48]. In the present study, fucoxanthin-treated HepG2 cells showed a significantly higher proteasome activity compared with the vehicle-treated cells. Western blot analysis revealed that fucoxanthin-down-regulated cyclin Ds were partially inhibited by pre-treatment with proteasome inhibitor, MG132. However, fucoxanthin also displayed a transcriptional repression of cyclin Ds mRNA in HepG2 cells. Taken together, it suggests that fucoxanthin caused the suppression of cyclin D through both promotion of proteasomal degradation and transcriptional repression and finally induced the cell cycle arrest. Previously we have reported that fucoxanthin inhibited the growth of human colon cancer WiDr cells through up-regulation of p21<sup>Waf1/Cip1</sup> without changes cyclin D level [25]. This differences may be due to variation in the endogenous growth factors produced in the different cell types, or variations in the growth-regulating signaling pathways altered in the different cell types during the process of immortalization and/or transformation.

In HepG2 cells we found that the expression of the cyclin dependent kinase inhibitor p21<sup>Waf1/Cip1</sup> was not influenced by the treatment with fucoxanthin. In agreement with our finding, Yoshiko *et.al.* [49] had previously found that in HepG2 cells fucoxanthin induces G<sub>0</sub>/G<sub>1</sub> arrest without significant changes in p21<sup>Waf1/Cip1</sup>. They hypothesized that *GADD45A*, p53-regulated gene interacting with the products of two different p53-regulated genes, p21<sup>Waf1/Cip1</sup> and proliferating cell nuclear antigen [50,51] is involved in fucoxanthin-induced G<sub>0</sub>/G<sub>1</sub> arrest. Since recently Ji and colleagues [52] reported in MEF cells that *GADD45A* induction is followed by cyclin D<sub>1</sub> suppression by

inhibiting the translocation of  $\beta$ -catenin to the nucleus. We hypothesize that also in HepG2 the observed fucoxanthin-induced decreased expression of cyclin D<sub>1</sub> may be driven by changes in *GADD45A* expression. Further investigations are required to clarify this point.

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## Figures legends

**Fig. 1.** Chemical structure of fucoxanthin.

**Fig. 2.** Fucoxanthin inhibited the growth of human hepatoma cells through cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> phase. (A) HepG2 cells were treated with the indicated concentration of fucoxanthin or 0.5% tetrahydrofuran (THF) as the vehicle control for different time points. Values were calculated as the percentage of the values in 0 h and represent the mean  $\pm$  SD (n= 5). \* Significantly different from the vehicle-treated cells at the corresponding time point. \*\* Significantly different from both the vehicle and 10  $\mu$ M fucoxanthin-treated samples at the corresponding time point. \*\*\* Significantly different from the vehicle-, 10, and 25  $\mu$ M treated at the corresponding time point. (B) HepG2 cells were treated with 25  $\mu$ M fucoxanthin or 0.5% THF as a vehicle control for 24 h. Values indicate the mean  $\pm$  SD (n=9). \* Significantly different from the vehicle treated at the corresponding time point.

**Fig. 3.** Fucoxanthin down-regulated the activity of the cyclin D/cdk 4 complex. (A) HepG2 cells were treated with 25  $\mu$ M fucoxanthin for the indicated time periods. The phosphorylation level of Rb was examined by western blotting analysis. (B) HepG2 cells were treated with 25  $\mu$ M fucoxanthin for 18 h. Immunoprecipitation kinase assay for the cyclin D/cdk4 complex was performed using an Rb-C fusion protein. Data shown is representative of three independent experiments. Rb, retinoblastoma protein.

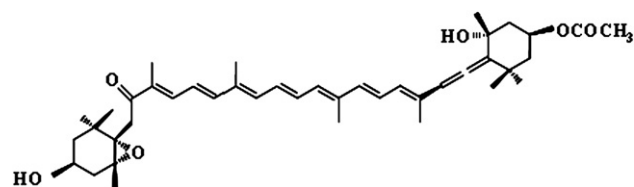
**Fig. 4.** Fucoxanthin caused down-regulation of D-type cyclins. (A) HepG2 cells were treated with 25  $\mu$ M fucoxanthin for the indicated time periods. Protein level of

cyclin D1, cyclin D3, and cdk4 was examined by western blotting analysis. (B) HepG2 cells were treated with 25  $\mu$ M fucoxanthin for the indicated time periods. Protein level of p27<sup>Kip1</sup> in fucoxanthin-treated HepG2 cells was examined by western blotting analysis.

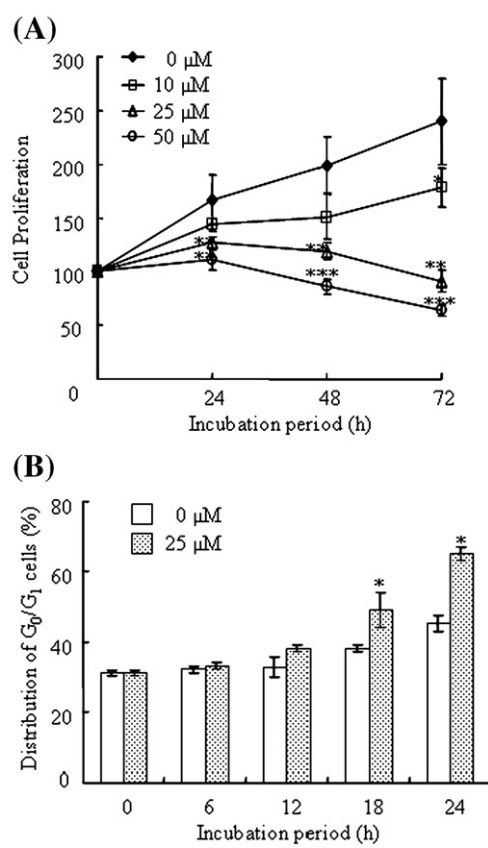
**Fig. 5.** Fucoxanthin induced the proteolysis of both cyclin D1 and cyclin D3.

(A) HepG2 cells were treated with 25  $\mu$ M fucoxanthin for 12 h, and proteasome activity was measured using a fluorogenic substrate, as described in Materials and Methods. The values represent the mean  $\pm$  SD (n= 3). \* Significantly different from the vehicle treated cells. (B) HepG2 cells were pre-treated with DMSO or proteasome inhibitor, MG132 (10  $\mu$ M), for 30 min and then treated with 25  $\mu$ M fucoxanthin. Protein level of cyclin D1 and D3 was examined by western blotting analysis. Data shown is representative of three independent experiments.

**Fig. 6.** Fucoxanthin decreased the expression of cyclin D1 and D3 mRNA in HepG2 cells. HepG2 cells were treated with 25  $\mu$ M fucoxanthin for the indicated time period. The mRNA expression levels of cyclin D1 and D3 are examined by RT-PCR.

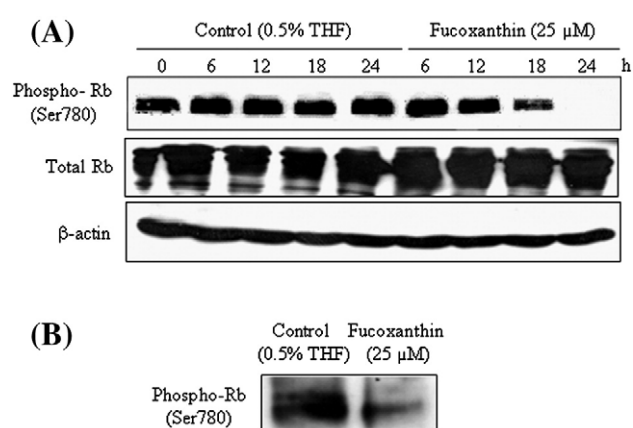


**Figure 1**

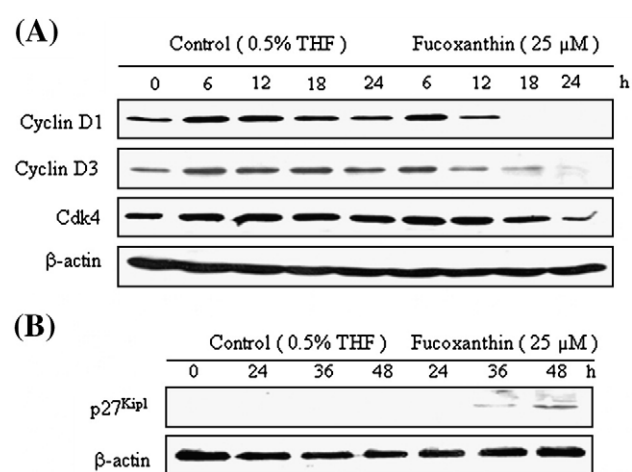


**Figure 2**

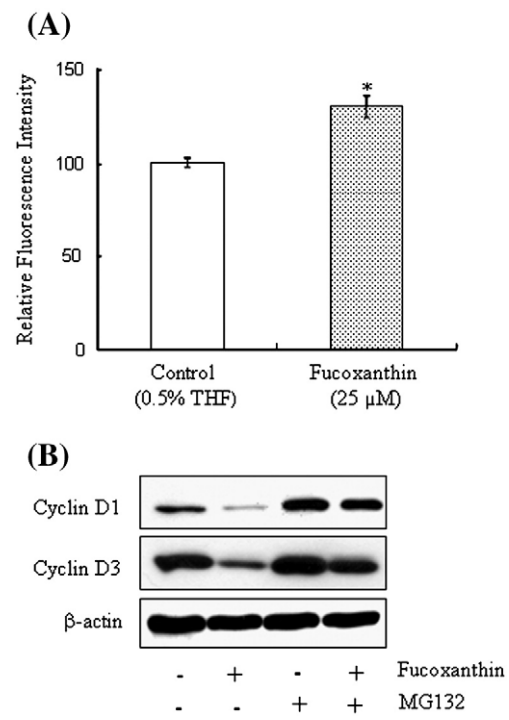




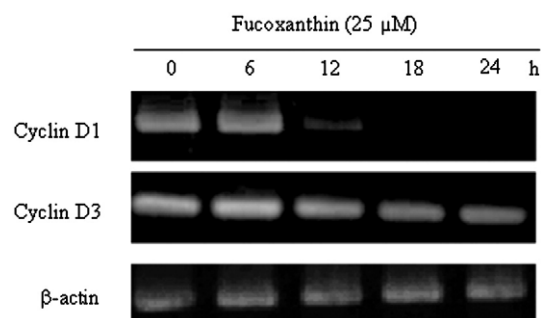
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**