

## Full Length Research Paper

# Effect of gene transfer of *Chlorella vulgaris* n-3 fatty acid desaturase on mouse breast cancer cells

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*Chlorella vulgaris* had the gene of n-3 fatty acid desaturase (CvFad3) which can synthesize the precursor of n-3 polyunsaturated fatty acids (PUFAs) or to convert n-6 to n-3 PUFAs. The objective of this study was to examine whether the CvFad3 gene from *C. vulgaris* can be functionally expressed in mammalian cells and whether its expression can exert a significant effect on cellular fatty acid composition. CvFad3 gene was inserted into plasmid pEGFP-C3 to construct eukaryotic expression vector pEGFP-C3-n-3 and expressed the n-3 Fad gene in mouse breast cancer cells (4T1 cells). Transfection of recombinant vector into 4T1 cells resulted in a high expression of n-3 fatty acid desaturase. Lipid analysis indicated a remarkable increase in the level of n-3 PUFAs accompanied with a large decrease in the contents of n-6 PUFAs. Accordingly, CvFad3 gene significantly decreased the ratio of n-6/n-3 PUFAs of 4T1 cells membrane. The expression of CvFad3 gene decreased cellular proliferation and promoted cellular apoptosis. This study demonstrates that CvFad3 gene could dramatically balance the ratio of n-6/n-3 PUFAs. It would be an effective approach to modifying fatty acid composition of mammalian cells and also provided a basis for potential applications of this gene transfer in experimental and clinical settings.

**Key words:** *Chlorella vulgaris*, CvFad3 gene, fatty acid desaturase, recombinant expression vector, fatty acid composition.

## INTRODUCTION

Biological effects of polyunsaturated fatty acids (PUFAs) have been widely investigated around the world. In particular, their impacts on human health have attracted increasing public attention in recent years. PUFAs mainly include two groups: n-3 PUFAs and n-6 PUFAs. The n-3 PUFAs have been the subject of increasing investigation and have attracted considerable interest as pharmaceutical compounds and nutraceuticals (Connor, 2000; Salem et al., 1996). N-3 PUFAs are beneficial for humans and animals and have been verified to exert preventive and therapeutic effects on some diseases such as cardiovascular diseases, arthritis, cancer and neuropathic diseases (Kris-Etherton et al., 2004; Mozaffarian et al.,

2005). Clinical Cancer Researches indicate that breast, and colon cancer can be modified or inhibited their growth by supplying n-3 PUFAs in human diet (Bougnoux et al., 1999; Rao et al., 2001). In general, a balanced n-6/n-3 ratio of the body lipids is essential for normal growth and development and plays an important role in the prevention and treatment of many clinical problems (Simopoulos, 2000). However, humans and mammals are incapable of synthesizing n-3 PUFAs in their bodies, so the levels of PUFAs in their bodies are, to a great extent, dependent on dietary intake (McLennan, 1998). Some plants, such as *Chlorella vulgaris* are able to synthesize the n-3 fatty acid. It is reported that a CvFad3 gene from *C. vulgaris* encoded the n-3 fatty acid desaturase (FAD), this enzyme, when expressed in *Nicotiana tabacum* can catalyze the conversion of n-6 PUFAs to n-3 PUFAs by introducing an n-3 double bond into their hydrocarbon chains (Suga et al., 2002). The objective of this study

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was to examine whether the Cv- Fad3 gene from *C. vulgaris* can be functionally expressed in mammalian cells in a high efficiency and whether its expression can exert a significant effect on cellular fatty acid composition.

## MATERIALS AND METHODS

### Construction of recombinant plasmid

The plasmid pEGFP-C3 (BD Bioscience Clontech) was digested by XhoI and EcoRI and fractionated on 1% agarose gels, then the long objective bands were purified. The fragment of CvFad3 gene was amplified from mRNA of *C. vulgaris* by reverse transcriptase-polymerase chain reaction (RT-PCR) and then inserted into the plasmid of pEGFP-C3 (Figure 1A). The detailed steps were described as follows: The long segment pEGFP-C3 and the CvFad3 gene were ligated in 1:4(mol/mol) by T4 ligase. The construct was confirmed by enzymatic digestion and DNA sequencing. The eukaryotic recombinant expression vector was named pEGFP-C3-n-3.

### Cell culture and transfection with recombinant plasmid

4T1 cells were obtained from Shanghai Life Science of Chinese Academy of Sciences. 4T1 cells were routinely maintained in 1:1 (v/v) mixture of DMEM high glucose and 10% (vol/vol) fetal bovine serum (FBS), 37°C in a tissue culture incubator with 5% CO<sub>2</sub> and 98% relative humidity. 4T1 cells were placed in 6-well plates and cultured as normal. The cells number and medium volume were the same in each well. After 24 h cultured, cells were transfected with recombinant plasmid pEGFP-C3-n-3 and pEGFP-C3-control for experiments when 75% of plate was covered by cells. Transfectants were carried out by adding X-fect polymer at the same culture medium with serum. After 5 h incubation, the transfection medium was replaced with normal culture medium. Forty-eight hours incubation cells were used for photographs, analysis of gene expression and fatty acid composition.

### MTT assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Roche Diagnostics Corporation) assay was performed to determine cell growth and viability. 4T1 cells ( $1 \times 10^4$  plaque-forming units per milliliter) were grown in 96 well culture plate. Two groups for pEGFP-C3-n-3 and pEGFP-C3-control were set up, and each group had 10 unions. After 48 h of transfection, 20  $\mu$ l MTT (5 mg/ml) labeling reagent was added to each well (100  $\mu$ l medium). The solution was then incubated with 50 mL/L CO<sub>2</sub>, at 37°C for 4 h. The medium was discarded and 100  $\mu$ l of the solubilization solution was then added into each well. Complete solubilization of the purple crystals was checked for and the spectrophotometric absorbency of the solution was measured at 490 nm.

### mRNA analysis

Briefly, total RNA was extracted from cultured cells transfected after 48 h by using a total RNA isolation reagent (TRIzol, Invitrogen), according to the manufacturer's protocol. Amplifying the CvFad3 gene fragment, reverse transcription-polymerase chain reaction (RT-PCR) was done. The forward and reverse primers were 5'-TTGCCGCTCCTGCCGAAGA-3' and 5'-GGGTCACCTGGGTCCGTGATGGT-3', respectively. The condition for amplification was 94°C for 3 min, 94°C for 30 s, 61°C for 40 s, 72°C for 1 min, 72°C

for 10 min. The amplification products were 793 bp. And the objective products were subjected to autoradiography.

### Gas chromatograph analysis

After 48 h transfection, the 4T1 cells were collected from 6-well plates. The fatty acid composition of total cellular lipids was analyzed as described (Weylandt et al., 1996; Kang et al., 1992). Lipid was extracted with chloroform/methanol (2:1, vol/vol) containing 0.005% butylated hydroxytoluene (BHT, as antioxidant). Fatty acid methyl esters were prepared by using a 14% (wt/vol) BF<sub>3</sub>/methanol reagent. Fatty acid methyl esters were quantified with gas chromatography/mass spectrometry (GC/MS) by using an HP-5890 Series II gas chromatograph equipped with a Supelcowax SP-10 capillary column (Supelco, Bellefonte, PA) attached to an HP-5971 mass spectrometer. The injector and detector are maintained at 260°C and 280°C, respectively. The oven program is maintained initially at 150°C for 2 min, then ramped to 200°C at 10°C/min and held for 4 min, ramped again at 5°C/min to 240°C, held for 3 min, and finally ramped to 270°C at 10°C/min and maintained for 5 min. Carrier gas-flow rate is maintained at a constant 0.8 ml/min throughout. Total ion monitoring is performed encompassing mass ranges from 50-550 atomic mass units. Fatty acid mass is determined by comparing areas of various analyzed fatty acids to that of a fixed concentration of internal standard.

### Flow cytometric analysis

After 48 h transfection, the 4T1 cells were collected from 6-well plates. Cell cycle and apoptosis were determined by Vybrant Apoptosis Assay Kit (Invitrogen), according to the manufacturer's protocol, and measured by fluorescence activated cell sorting using a FACScan flow cytometer (Becton Dickinson).

### Data analysis

Cell growth and viability data (MTT), fatty acid composition level was analysed by GraphPad Prism 5 software. The figures of cell cycle and apoptosis were made by WinMDI 2.9 software, each experiment was done 3 times. All data were expressed as the mean  $\pm$  standard deviation (SD) and were analyzed using the Student's t-test. The level of significance was set at  $P < 0.05$ .

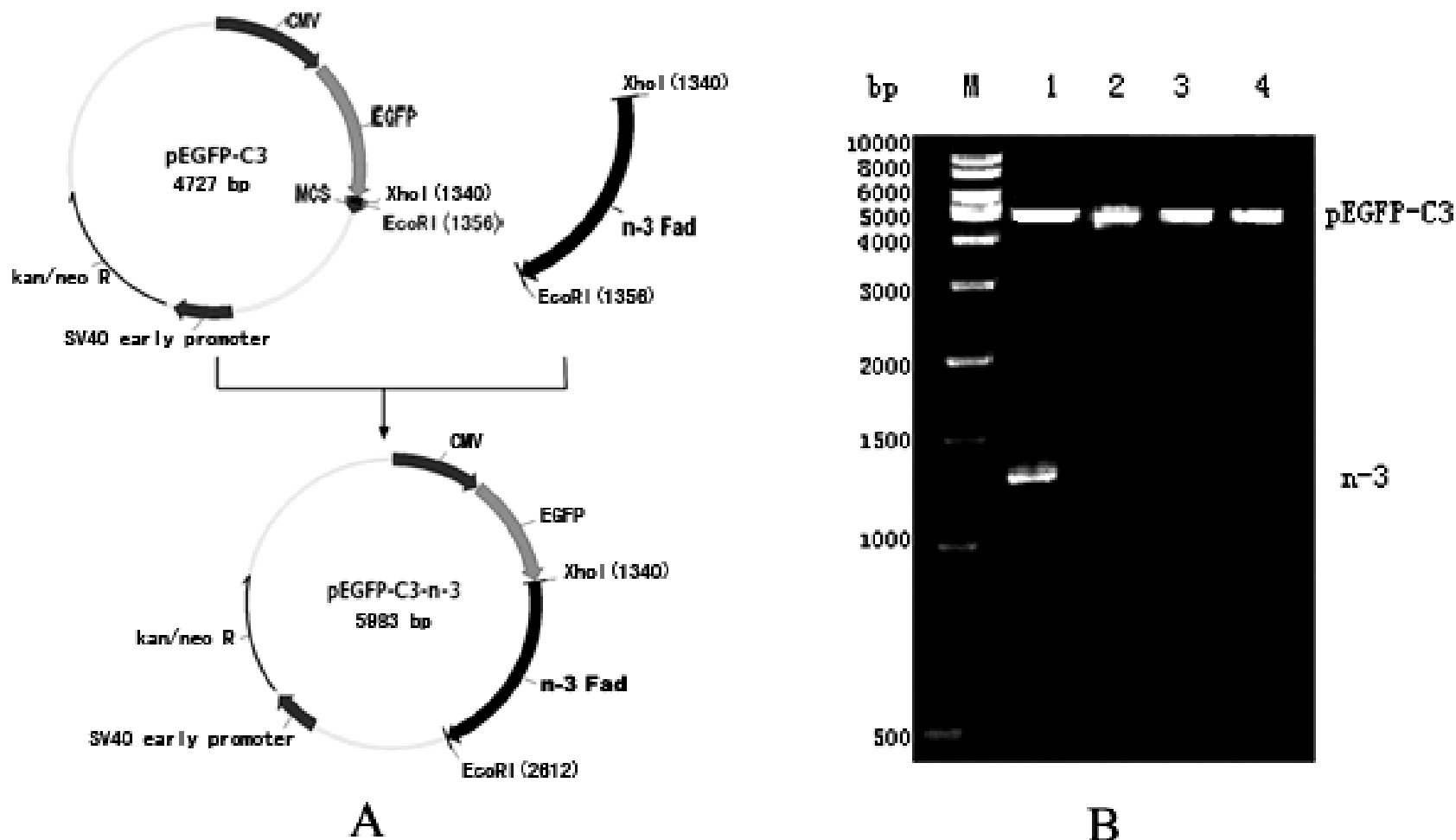
## RESULTS

### Enzymatic digestion of recombinant plasmid

Recombinant plasmid pEGFP-C3-n-3 was digested by the fast-digest Xho I and EcoR I, the result corresponded to plasmid pEGFP-C3 and the objective CvFad3 gene (Figure 1B).

### Expression of CvFad3 gene in 4T1 cells

The co-expression of EGFP allowed us to identify the cells that were transfected and expressed the transgene. The result is shown in Figure 3, 48 h after transfection about 40% of the cells exhibited bright fluorescence, indicating a high efficiency of gene transfer and a high expression level of the transgene. The cells expressed

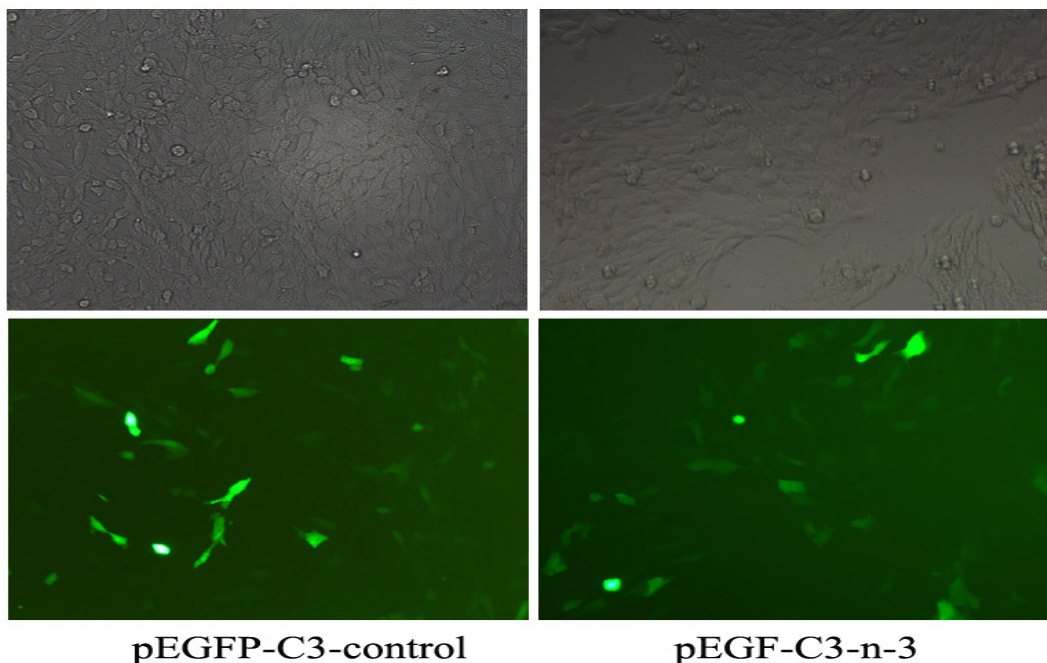


**Figure 1.** Construction and identification of recombinant plasmid pEGFP-C3-n-3. A: Construction of recombinant plasmid pEGFP-C3-n-3. The plasmid pEGFP-C3 was digested by XhoI and EcoRI, and the long objective bands were purified. The fragment of CvFad3 gene was amplified from mRNA of *Chlorella vulgaris* by RT-PCR and then inserted into the plasmid of pEGFP-C3. B: The identification of recombinant plasmid pEGFP-C3-n-3 and pEGFP-C3 by double enzyme XhoI/EcoRI digestion. M 1kb DNA ladder; 1 2 3 XhoI/EcoRI, digest pEGFP-C3-n-3; 4 XhoI/EcoRI digest pEGFP-C3. Products of PCR were observed by 1.0% agarose electrophoresis.

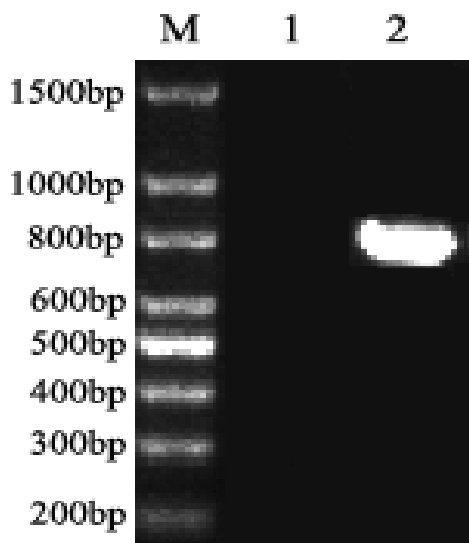
CvFad3 gene mostly died, but few cells died in the cells only expressed EGFP gene, as shown in Figure 2. Furthermore, expression profile of the

transgene also was determined by RT-PCR. As shown in Figure 3, the mRNA of CvFad3 gene was not detected in cells transfected with pEGFP-

C3-control but was highly abundant in cells transfected with pEGFP-C3-n-3. The results indicate that CvFad3 gene has a very high



**Figure 2.** Photomicrographs showing gene-transfer efficiency. 4T1 cells were transfected with pEGFP-C3-control or pEGFP-C3-n-3. Forty-eight hours after tranfection, the group of pEGFP-C3-control was brighter than the group of pEGFP-C3-n-3. The cells expressed CvFad3 gene mostly died, but few cells died in the cells only expressed EGFP gene.



**Figure 3.** Level of n-3 FAD transcript in 4T1 cells transfected with pEGFP-C3-control and pEGFP-C3-n-3 after 48 h. Lane M 100 bp DNA ladder; lane 1 pEGFP-C3 -Control group; 2 lane pEGFP-C3-n-3-group. Products of PCR observed by 2.0% agarose electrophoresis. This result indicates that: CvFad3 mRNA was not detected in 4T1 cells transfected with pEGFP-C3-Control, but was highly abundant in 4T1 cells transfected with pEGFP-C3-n-3.

Expression in 4T1 cells transfected with pEGFP-C3-n-3.

### Effect of n-3 desaturase on fatty acid composition

We tested whether the expression of CvFad3 gene in the 4T1 cells can lead to conversions of n-6 fatty acids to n-3 fatty acids and, thereby, a change in fatty acid composition. The fatty acid composition of total cellular lipids was analyzed by gas chromatograph. These results are summarized in Table 1. The fatty acid profiles are remarkably different between the control cells transfected just with the *pEGFP-C3* and the cells transfected with the *pEGFP-C3-n-3*. In the cells expressing the CvFad3 gene, n-6 fatty acids were converted largely to the corresponding n-3 fatty acids, namely, 18:2n-6 to 18:3n-3, and 20:4n-6 to 20:5n-3. As a result, the fatty acid composition of the cells expressing the n-3 fatty acid desaturase was changed significantly when compared with that of the control cells transfected with *pEGFP-C3-control*. Importantly, the ratio of n-6/n-3 was reduced from 6:1 in the control cells to 1:1.2 in the cells expressing the n-3 fatty acid desaturase.

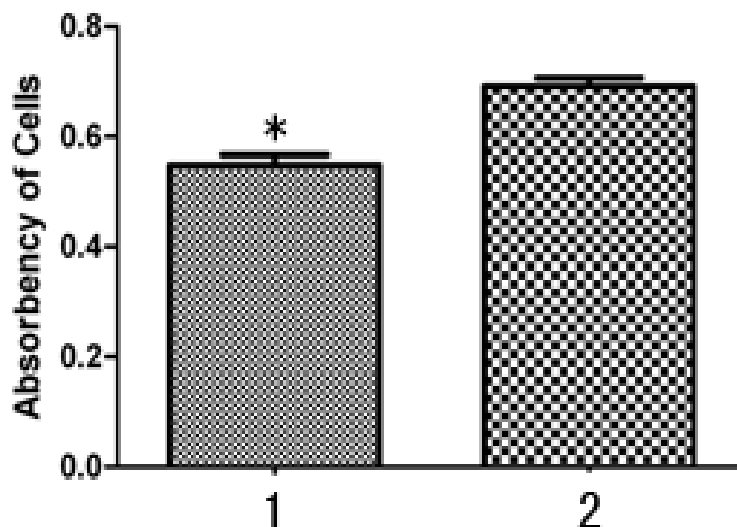
### Change of cellular proliferation

To investigate the effect of expression of CvFad3 gene on 4T1 cells growth, we analyzed proliferation of cells. As shown in Figure 4, in the result of MTT kit (cell growth and viability assay), the absorbency of control cells was higher than that of experiment group. That is to say, the growth and viability of control cells was higher than that

**Table 1.** PUFA composition of total cellular lipids from the control 4T1 cells and the transgenic cells expressing CvFad3 gene (area percentage,  $\bar{X} \pm s$ , n = 3).

| PUFAs composition | Control group | Experiment group |
|-------------------|---------------|------------------|
| <b>n-6 PUFAs</b>  |               |                  |
| 18:2 n-6          | 7.27±0.134    | 2.13±0.049*      |
| 20:4 n-6          | 4.73±0.064    | 1.85±0.052*      |
| 22:5 n-6          | 1.62±0.041    | 0.86±0.039*      |
| Total             | 13.62         | 4.84             |
| <b>n-3 PUFAs</b>  |               |                  |
| 18:3 n-3          | 0.95±0.047    | 2.93±0.128*      |
| 20:5 n-3          | 0.75±0.043    | 2.04±0.051*      |
| 20:6 n-3          | 0.46±0.039    | 1.07±0.024*      |
| Total             | 2.16          | 6.04             |
| n-6/n-3 ratio     | 6.31          | 0.80             |

Partial gas chromatograph determined fatty acid profiles of total cellular lipids extracted from the control cells transfected with pEGFP-C3-Control and the cells transfected with pEGFP-C3-n-3. The fatty acid profiles are remarkably different between the control cells transfected just with the pEGFP-C3 and the cells transfected with the pEGFP-C3-n-3. In the cells expressing the CvFad3 gene, n-6 fatty acids were converted largely to the corresponding n-3 fatty acids, namely, 18:2n-6 to 18:3n-3, and 20:4n-6 to 20:5n-3. \* $P < 0.01$  compared to control group.

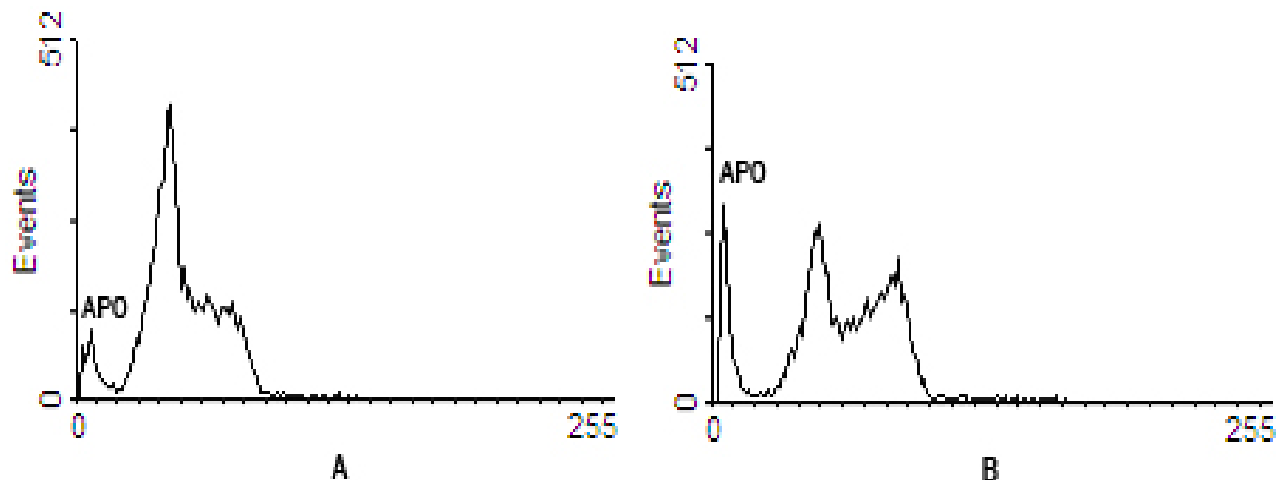


**Figure 4.** The proliferation of 4T1 cells (the control cells and the cells expressing n-3 fatty acid desaturase) were assessed by MTT. Note: 1: pEGFP-C3-n-3 group 2: pEGFP-C3-Control group. \* $P < 0.01$  compared to control group. The absorbency of cells transfected with pEGFP-C3-n-3 was lower than that of the cells transfected with pEGFP-C3-Control.

of experiment cells with CvFad3 gene, because the more live cells or less of dead cells are, the higher the absorbency of cells is in 490 nm ( $P < 0.01$ ). This indicates that the expression of CvFad3 gene can cause 4T1 cells to die and/or inhibits the growth, leading to decrease of its cellular proliferation.

#### Change of cell cycle and apoptosis

Compared with cells transfected with pEGFP-C3-control, in the 4T1 cells transfected pEGFP-C3-n-3, the percent of  $G_0/G_1$  period was lower ( $P < 0.01$ ); while the percent of  $G_2/M$  period was higher ( $P < 0.01$ ); the percent of S period



**Figure 5.** Change of cell cycle. Note: A: pEGFP-C3 –Control group B: pEGFP-C3-n-3 group. Compared with cells transfected with pEGFP-C3-control, in the 4T1 cells transfected pEGFP-C3-n-3, the percent of G<sub>0</sub>/G<sub>1</sub> period was lower ( $P<0.01$ ); while the percent of G<sub>2</sub>/M period was higher ( $P<0.01$ ); the percent of S period and APO period obviously increased (both  $P<0.01$ ).

and APO period obviously increased (both  $P<0.01$ , Figure 5). These results indicate that CvFad3 gene could inhibit 4T1 cells proliferation, make cell cycle blocked at G<sub>2</sub>/M period, and cells could not cleavage subsequently. Apoptosis analysis showed that compared with cells transfected with pEGFP-C3-Control, in the 4T1 cells transfected with pEGFP-C3-n-3-control, the percent of early apoptosis and late apoptosis were higher ( $P<0.01$ , Figure 6).

## DISCUSSION

In this study, we expressed the construct in 4T1 cells to estimate the product activity as well as expression of CvFad3 gene in mammals. This study clearly demonstrated that the CvFad3 gene from *C. vulgaris* can be expressed functionally in mouse breast cancer cells, and its expression could confer cells' capability of converting n-6 PUFAs to corresponding n-3 PUFAs, leading to a balanced n-6/n-3 ratio, that could inhibit cell growth and induce apoptosis in breast cancer cells.

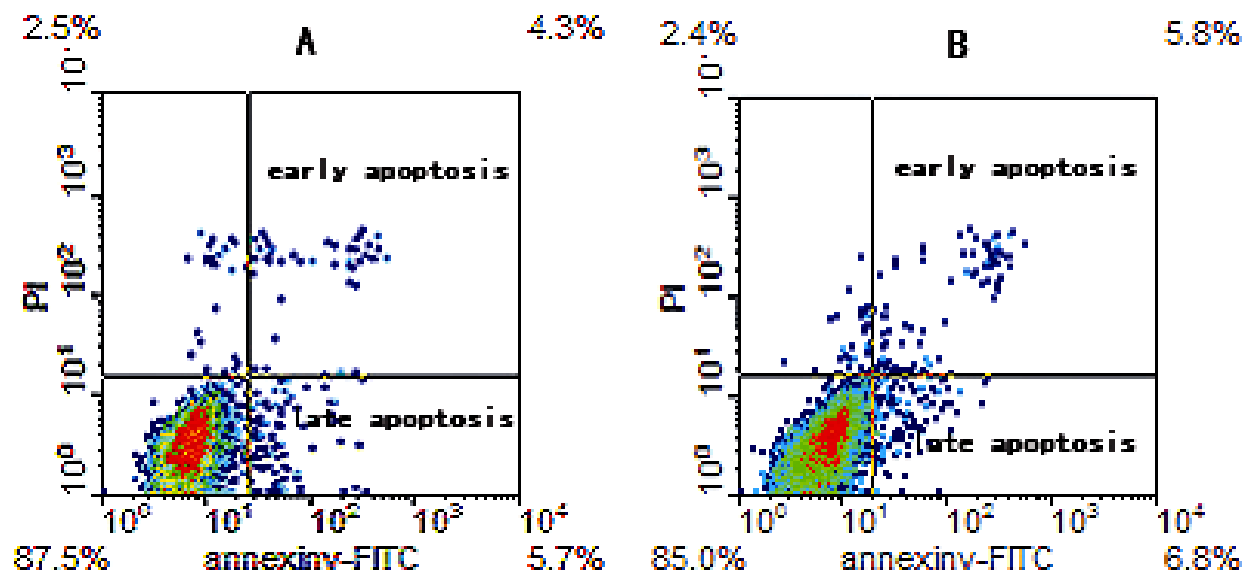
We have known that n-6 PUFAs stimulate animal's tumor and cancer growth and metastasis, whereas n-3 PUFAs exhibit inhibitory effects. A number of studies have shown n-3 fatty acid protection in rodent models of breast cancer. These include dietary supplementation in mouse transplantable tumors (Gabor et al., 1985) and human cell xenograft models (Rose et al., 1995) as well as chemically induced tumors in rats (Takahashi et al., 1994). Many signaling pathways that are relevant to carcinogenesis and tumor progression are differentially affected by n-3 and -6 PUFAs.

For instance, n-6 PUFA products were reported to upregulate and activate cellular signaling mediators including protein kinase C, ras, ERK 1/2 and NF- $\kappa$ B

whereas n-3 PUFA products had the opposite effect. The ability of long-chain n-3 PUFAs to induce apoptosis in tumor cells has also been attributed to the increased susceptibility of these cells to lipid peroxidation (Stoll, 2002). Inhibition of tumor cell growth and invasion by n-3 PUFAs in a xenograft animal model was associated with decreased COX-2 and PGE<sub>2</sub> levels (Kobayashi et al., 2006). Thus, n-3 PUFAs may act as a natural COX "inhibitor".

It is a significant method for controlling tumor development to balance the ratio of n-6/n-3 PUFAs (Simopoulos et al., 2002). Meanwhile, the n-3 and n-6 PUFAs are not interconvertible in the human body, because mammalian cells lack the n-3 fatty acid desaturase. With hectic schedule, poor dietary habits and low quality of food sources that people have today, experts recommend the consumption of nutritional dietary supplements, including fish oil supplements, which help fulfill the nutritional demand of all body organs (decrease the intake of n-6 fatty acids and increase the intake of n-3 fatty acids) (Simopoulos et al., 1999). Fish oil is oil derived from the tissues of deep-sea oily fish, but this oily fish does not actually produce docosahexaenoic acid/eicosapentaenoic acid (DHA/EPA) fatty acids, but instead accumulate them from consuming deep-sea micro-algae that produce these fatty acids. *C. vulgaris* is one kind of deep-sea micro-algae, and it has the CvFad3 gene, encoding n-3 FAD which can catalyze the conversion of n-6 PUFAs to n-3 PUFAs.

Therefore, an alternative approach that can quickly and effectively increase cellular n-3 PUFA contents and balance the n-6/n-3 ratio, without the need for a lengthy intake of fish oil supplements would be desirable. Because lack of an n-3 desaturase gene is the bottleneck for endogenous production of n-3 PUFAs in mammals, transfer of the n-3 desaturase gene from deep-sea micro-



**Figure 6.** Change of cell apoptosis. Note: A: pEGFP-C3 –Control group B: pEGFP-C3-n-3 group. Compared with cells transfected with pEGFP-C3 –Control, in the 4T1 cells transfected pEGFP-C3-n-3–control, the percent of early apoptosis and late apoptosis were higher ( $P<0.01$ ).

algae into livestock is the key to overcoming this constraint, as these animals already possess most of these other desaturases and PUFA elongases (Wallis et al., 2002).

This assumption has been shown to be feasible in transgenic mice generated with the n-3 desaturase gene from the roundworm *C. elegans*. A transgenic mouse model expressing the fat-1 gene increased the ratio of n-3 and n-6 fatty acids in various tissues (Kang et al., 2004). In this study, we used eukaryotic recombinant expression vector to transfer the CvFad3 gene from *C. vulgaris* into mouse breast cancer cells, convert n-6 PUFA into n-3 PUFA to increase the level of n-3 PUFA and the ratio of n-3/n-6 PUFA. Our experimental results showed that the transgenic construct expressed the interest protein and the transgene product possessed its biological activity in the transfected mammal cells. Then, we demonstrated that the CvFad3 gene from *C. vulgaris* was functionally expressed and that its product has a significant effect on the fatty acid composition. The proportion of n-6 PUFAs decreased and n-3 PUFAs increased considerably, particularly for ALA, DHA and EPA, which correspondingly inhibited cell growth and induced apoptosis in breast cancer cells. All these results showed that the CvFad3 gene from *C. vulgaris* possesses great value for the production of n-3 PUFAs and cancer cell suppression in transgenic 4T1 cells.

Our findings as presented here suggest that gene transfer of the CvFad3 gene from *C. vulgaris* could be such a desirable intervention that can quickly and effectively provide therapeutic and cancer-preventive effects of n-3 fatty acids. The molecular mechanisms

which account for these biological effects are not completely understood. Certainly, further study in this regard is warranted.

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