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*Full Length Research Paper*

# Establishment and comparison of three different codon optimization of *fat1* gene in transgenic mammary epithelial cell lines

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***Fat1* gene is an n-3 fatty acid encoding desaturase from *Caenorhabditis elegan* (*C. elegan*). It can raise the n-3/n-6 polyunsaturated fatty acids (PUFAs) ratio in mammalian cells. To reveal the impact of different codon optimizations of *fat1* gene in influencing the catalysis efficiency of n-6 PUFAs into n-3 PUFAs in mammalian cells, the *fat1* gene from *C. elegan* coding sequence was optimized based on the codon usage frequency preference of bovine muscle protein, milk protein and the mixtype, named as r*fat1*, n*fat1* and y*fat1*, respectively. Three different codon optimized *fat1* gene mammalian cell expression vectors were constructed and transfected into mammary epithelial cells of Chinese Holstein Cows. As expected, the specific fragment was amplified by RT-PCR from the transfected cells. Also, different catalysis efficiencies were observed with different codon optimizations. This study will help to understand the impact of codon usage bias, and the mechanisms of tissue specific regulation of gene expression of *fat1* on bovine mammary epithelial cell lines.**

**Key words:** *Fat1*, codon usage bias, gene expression, mammary epithelial cells.

## INTRODUCTION

Synonymous codons are not used with equal frequencies (Comeron and Aguade, 1998). The over abundance in the number of codons allows many amino acids to be encoded by more than one codon because there are 64 different codons (61 codons encoding for amino acids plus 3 stop codons) for only 20 different amino acids. Different tissues and organisms often show particular codon usage bias for the same amino acid. Codon usage is different in different genomes as well as in the same genome (Grantham et al., 1980a, 1980b). It means that a higher frequency of one will be found than expected purely by chance. How such preferences arise is a much debated area of molecular evolution. It is generally acknowledged that codon preferences reflect a balance between mutational biases and natural selection for translational optimization, and proposed to be related by different factors (Ermolaeva, 2001).

N-3 polyunsaturated fatty acids (PUFAs) have beneficial effect on human health (Lopez-Huertas, 2010), while a rational balanced level of n-6 and n-3 PUFAs is important and the ideal n-6/n-3 ratio is 1:1 (Enser et al., 1996, 1998). In terms of human being, however, it has enzyme to neither synthesize n-6 and n-3 fatty acids nor convert n-6 to n-3 fatty acids (Spychalla et al., 1997). n-3 Fatty acid desaturase contains 402 amino acids which can transform C18 and C20 n-6 PUFAs into n-3 PUFAs. *Fat1* gene from *Caenorhabditis elegan* (*C. elegan*) encodes a n-3 fatty acid desaturase. The first *fat1* transgenic pig which can produce high levels of n-3 fatty acids from n-6 analogs was produced in 2006 (Lai et al., 2006), but the concentration of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA), the so-called highly unsaturated fatty acids (HUFAs) which play a key role in growth and development, was still with a low level

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**Table 1.** Primers of different x*fat1* gene and GAPDH control.

| Primer | Sequence | Digest enzyme site |
|---|---|---|
| r*fat1*-F | ACTTCTAGAGCCACC*ATG*GTCGCTCATTCC | *Xba*I |
| r*fat1*-R | ACTGCTAGC*TTA*CTTGGCCTTTGCCTTCT | *Nhe*I |
| n*fat1*-F | ACTGACGTC*ATG*GTTGCCCACAGTAGT | *Aat* II |
| n*fat1*-R | ACTTCTAGA*TTA*CTTGGCCTTGGCCTT | *Xba* I |
| y*fat1*-F | ACTGACGTC*ATG*GTCGCTCACTCC | *Aat* II |
| y*fat1*-R | ACTTCTAGACCTCGAG*TTA*CTTGGCCT | *Xba* I |
| GAPDH-F | TGGTATCGTGGAAGGACTCATGAC | |
| GAPDH-R | ATGCCAGTGAGCTTCCCGTTCAGC | |

Underscore indicates digest enzyme sites; italic indicates initiation/stop codon.

(Olivotto et al., 2011).

Zhu et al. (2008) cloned another *fat1* gene from *Caenorhabditis Briggsae* (GenBank accession number: CAAC01000009) and called it *sFat1*. The results showed that *sFat1* produce a marked effect for transforming n-6 PUFAs into n-3 PUFAs, including more long-chain HUFAs like EPA, DHA and DPA (Zhu et al., 2008). The *sFat1* gene is similar to *fat1*, with 71.5% gene sequence identity and 86.1% amino acids similarity with the absence of two amino acids. Meanwhile, n-3 fatty acid desaturases from other species, such as *Mortierella alpina* and *Saprolegnia diclina* (Pereira et al., 2004; Sakuradani et al., 2005) have been studied, but no conserved cDNA region is found.

In the present study, we have optimized three types of *fat1* gene coding sequence based on the codon usage frequency preference of bovine muscle protein, milk protein and the mixtype, and named them r*fat1*, n*fat1* and y*fat1*. We then inserted these three different codon optimized *fat1* genes into the mammalian cell expression vector pEF-GFP-IRES and transfected into mammary epithelial cells (MEC) of Chinese Holstein Cows. Comparison of the catalysis efficiency could provide scientific insights into the regulation of codon usage bias and the mechanism of fatty acid and milk protein metabolism in bovine mammary epithelial cells. The research of the expression efficiency of *fat1* gene in MEC cells will help to gain the best cell nuclear donor for the production of transgenic *fat1* cattle.

## MATERIALS AND METHODS

### Codon optimization of *fat1* gene

In order to study which type of *fat1* gene codon had the highest expression in mammary epithelial cells (MEC) and to select the best optimization approach, we optimized the codon of *fat1* sequence from *C. elegan* based on the bovine milk protein, muscle protein and a mixed approach codon usage frequency preference (mixtype) (called n*fat1*, r*fat1* and y*fat1*, respectively) without any changing of amino acid sequence. DNA coding sequences of bovine muscle and milk proteins were analyzed by CHIPS (codon heterozygosity in a protein coding sequence) of EMBOSS (the European molecular biology open software suite) (Marra et al., 2003; Wright, 1990), GeneQuest from DNAStar software and codon usage of Sequence Manipulation Suite (SMS) (http://www.bio-soft.net/sms/index.html) (Stothard, 2000). The effective numbers of codons (Frank Wright Nc value) could help to judge the numbers of codon types. High level expression gene has a low Nc value, by contrast, high Nc value means low level express efficiency. Codon optimized *fat1* was synthesized by Sangon Biotech Company (Shanghai, China).

### Construction of vector

In this mammalian cell expression vector, named pEF-GFP-*fat1*, a codon optimized *fat1* cDNA was driven by the elongation factor 1-alpha promoter (EF1α promoter). An enhanced green fluorescent protein (eGFP) gene was also expressed through an internal ribosome entry site (IRES) sequence, and a neo-kan and amp expression cassette as selection marker. *Fat1* gene was cloned from the original vectors pUC57 using primers listed in Table 1. The eukaryotic expression vector pEF-GFP (Addgene plasmid NM 11154) was provided by Prof. Hongsheng Ouyang (Jilin University, China). For quantifying the positive cell lines, we designed and synthesized the A209 sequence, which contains a neo, IRES, and an eGFP. The A209 fragment was cloned into the pEF-GFP vector to replace the original eGFP sequence and named pEF-GFP-IRES. Then, the expression cassette of *fat1* was cloned into pEF-GFP-IRES vector to complete the resulting construct pEF-GFP-x*fat1* (x means different optimized approach).

### Cell culture and cytotoxicity assay

MEC cells of Chinese Holstein Cows were provided by the author's laboratory, which were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; PAA, Austria), 1% (v/v) L-glutamine (Gibco, USA) and penicillin (100 U/ml) and streptomycin (100 µg/ml) (PAA, Austria), at 37°C, 5% $CO_2$. DMEM/F12 with different concentrations of G418 antibiotic (300, 400, 500, 600, 700, 800 µg/ml, respectively) was prepared as selection medium and used to replace growth medium. The cells were incubated to proliferate for 15 days under selection medium, and the lowest concentration of G418 which can cause cells' entire survival rate to 0% were defined as the optimum concentration. The

**Table 2.** Frank Wright Nc value of x*fat1* and *C. elegans fat1* calculated by CHIPS programs.

| Name | n*fat1* | r*fat1* | y*fat1* | *C. elegans fat1* |
|---|---|---|---|---|
| Nc Value | 20.000 | 34.588 | 38.350 | 41.598 |

tests were performed in triplicate.

#### Transfection and selection of mammal epithelial cells

One day before transfection, 0.5 to 2 × $10^5$ MEC cells were plated into 500 µl of culture medium without antibiotics per well of a 6-well culture plate. When the cells reached more than 90% confluency, the culture medium was replaced by Opti-MEM serum-free media (Gibco, USA). Cells were transfected with 2 µg plasmid DNA using FuGENE HD Transfection Reagent (Roche, Germany) according to the manufacturer's protocol. The cells were incubated at 37°C in a $CO_2$ condition. Within 18 h, the selection DMEM/F12 containing 500 µg/ml of G418 antibiotic for an additional 12 days was change. Cells were propagated and split into selection DMEM/F12. The surviving cell colonies were picked and propagated in a new plate until stable cell lines formation. pEF-GFP-IRES vector transfected cell lines were used as the control, and performed under the same conditions (n = 3).

#### Analysis of transfected cells by RT-PCR

Expression of bovine *fat1* mRNA on MEC cells were determined by RT-PCR. On the 12th day, cells were collected and pelleted; total RNA was extracted from positive cells and cDNA was prepared using Oligo dT. The cDNAs were used as templates and specific primers were used to amplify the *fat1* coding region; GAPDH primers were used as a control. Primers used in RT-PCR reactions were given in Table 1. PCR was performed in a 25 µl reaction system with 35 cycles: 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. The PCR products were subjected to electrophoresis on a 1.5% agarose gel.

#### Real-time RT-PCR assessment of the expression of three different *fat1* genes

x*fat1* gene mRNA expression was analyzed on Eppendorf Mastercycler ep realplex System by fluorescent dyes SYBR Green real-time RT-PCR, data from which were computed by comparative $C_T$ method while GAPDH was chosen as control (sequences shown in Table 1). The conditions used for Real-time RT-PCR were 2 min at 95°C and then 40 cycles of 15 s at 95°C and 40 s at 60°C. For each experiment, pEF-GFP-IRES vector and untransfected cells were included as negative controls. Each cDNA sample was tested in duplicate (n = 3), and the mean values were calculated.

#### Catalysis efficiency analysis by gas chromatograph

Gas chromatograph (GC) was used to analyze the catalysis efficiency of *Fat1* protein. Mixed clones were mixed in order to eliminate the effects of the gene insertion sites. Lipids were extracted as previously reported (Kang and Wang, 2005; Lai et al., 2006; Lu et al., 2008). Briefly, cell pellet was collected in a glass methylation tube, mixed with 4.0 ml MeOH and 2.0 ml trichloromethane (capillary GC; Sigma-Aldrich, USA), 1.5 ml $ddH_2O$, and incubated on a rotating platform shaker for 10 s. After 15 min at RT, trichloromethane and $ddH_2O$ were added and mixed again, left at RT for 5 min, then vortexed and centrifuged, the lower phase was dried under nitrogen and re-suspended in boron trifluoride methanol, 1.5 ml of 14% $BF_3$/MeOH reagent was then added, after mixing vigorously, the mixture was heated at 90°C for 30 min, and cooled to room temperature. The mixture was vortexed again and the upper phase was recovered. 1.5 ml $ddH_2O$ and 4.0 ml hexane was then added and the methyl esters were extracted in the hexane phase. The samples were centrifuged for 1 min, and then the upper hexane layer was removed and concentrated under nitrogen.

Fatty acid methyl esters were analyzed by gas chromatography using a fully automated HP5890 system (Agilent) equipped with a flame-ionization detector. The extracts were dried, re-suspended, and injected into a capillary column SP-2560 (Supelco), 100 m × 0.25 mm ID, 0.20 µm film thicknesses (Sigma-Aldrich, USA). Identification of components was done by comparison of retention times with those of authentic standards (Sigma-Aldrich, USA).

#### Statistical analysis

All experiments were performed three times and representative results were shown. While data were shown as the mean ± standard deviation (SD) for indicated number of experiments. Bonferroni's Multiple Comparison Test was used to evaluate statistical significances between different treatment groups in Real-time RT-PCR assessment, Chi-square test was used to evaluate statistical GC analysis, with $p < 0.05$ considered statistical significance.

## RESULTS

### Codon optimization and sequence analysis of *fat1* gene

In n*fat1*, r*fat1* and y*fat1*, we changed 259, 65 and 51 bases, respectively, which affect 215, 65 and 51 codons, respectively. The lengths of n*fat1*, r*fat1* and y*fat1* were 1221, 1234 and 1221 bp, respectively. The results showed that all the sequences of three types of *fat1* were beneficial to the expression of the gene. The prediction result of the RNA secondary structure by DNAStar GeneQuest showed that the optimized gene could have high level expression with low minimum energy and non-crowd gene structure. The Frank Wright Nc value of each optimized *fat1* was calculated using CHIPS programs and the results showed that there was a decreasing of Frank Wright Nc value after optimization (Table 2). The *fat1* CDS sequence after optimization could be favorable to express in specific tissues.

### Identification of pEF-GFP-x*fat1* vectors

Correct pEF-GFP-x*fat1* vector was identified by

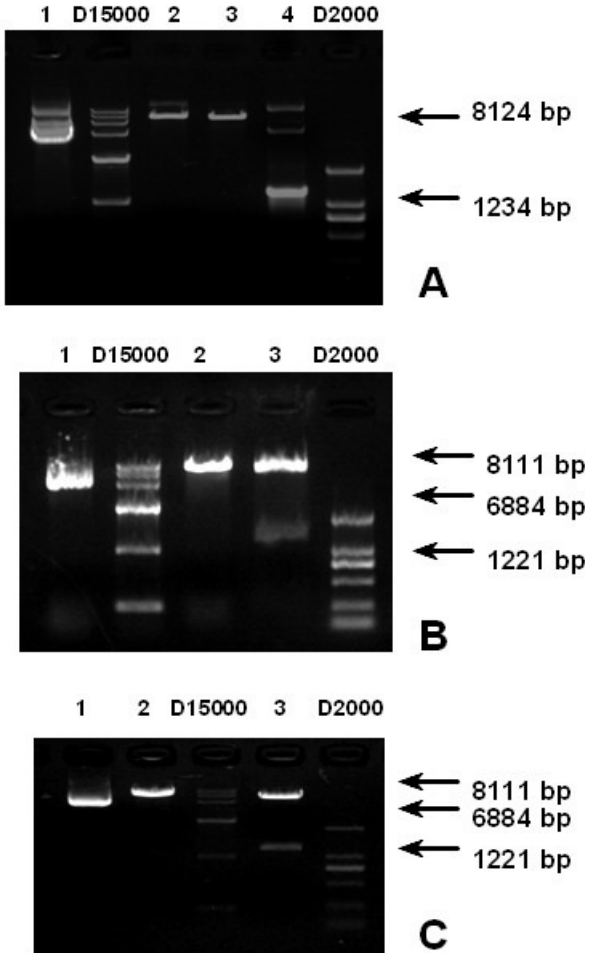


**Figure 1.** Identification of pEF-GFP-x*fat1* using enzyme digests and PCR. A: pEF-GFP-r*fat1*. 1, pEF-GFP-r*fat1* plasmid; 2, digestion by restrictive enzyme *AatII*; 3, digestion by restrictive enzyme *XbaI*; 4, PCR selective amplification r*fat1*. B: pEF-GFP-n*fat1*. 1, pEF-GFP-n*fat1* plasmid; 2, digestion by restrictive enzyme *XhoI*; 3, double digestion by restrictive enzyme *AatII*/ *XbaI*; C: pEF-GFP-y*fat1*. 1, pEF-GFP-y*fat1* plasmid; 2, digestion by restrictive enzyme *XhoI*; 3, double digestion by restrictive enzyme *AatII*, *XbaI*.

restriction enzyme digestion, PCR and sequencing. The multi cloning sites of *Aat*II / *Xba*I (TaKaRa, China) were incorporated into the *fat1* gene sequence during synthesis, and an *Xho*I site was inserted into the pEF-GFP-IRES vector. The expected fragments were obtained by complete digestion of the pEF-GFP-x*fat1* plasmid. The target fragment coding region of *fat1* gene was amplified from pEF-GFP-x*fat1* plasmid with specific primers by PCR. The results showed that the target gene fragment was successfully inserted to the pEF-GFP-IRES vector.

The constructed plasmids were transformed and amplified in *E. coli* (TOP10, Tiangen, China) and extracted using AxyPrep Plasmid Miniprep Kit (Axygen, China) from positive clones, then sequenced by Beijing Genomics Institute (China). The sequence was aligned by DNAStar and BLAST. The results showed that these three *fat1* gene was successfully cloned into *Aat*II / *Xba*I sites of the pEF-GFP-IRES vector (Figure 1).

## Cytotoxicity test of G418 in mammal epithelial cells

After 3 days of selection with different concentrations of

**Table 3.** Cytotoxicity test of G418 to cultured cells for 12 day.

| G418 concentration (μg/ml) | 300 | 400 | 500 | 600 | 700 | 800 |
|---|---|---|---|---|---|---|
| Survival rate (%) | + | + | – | – | – | – |

+, Survival rate of ≥ 30%. –, survival rate of 0%.

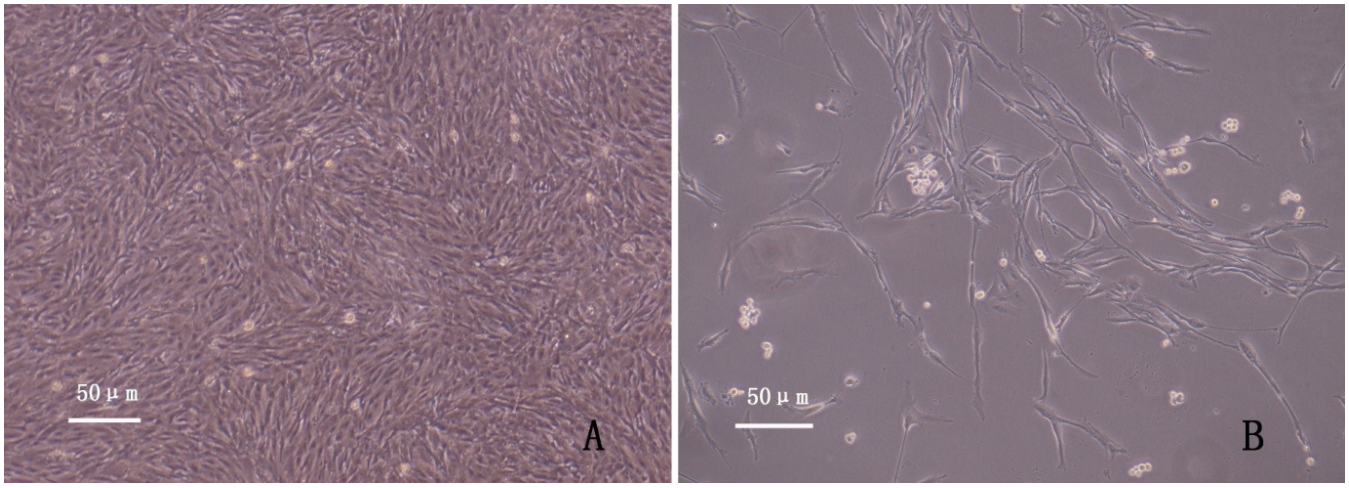


**Figure 2.** Compare normal cells with positive cells in morphological, under microscope (40×). A: normal cells before transfection. B: Positive cell aggregate, 12 days after G418 selected. The pictures show that positive cell aggregates continue growing while others died after selected.

G418, each group of MEC cells were found to be in various degrees of death, and the peak mortality was in 8 to 10 days. At day 12, cells treated with 600 μg/ml G418 or more were dead completely, as shown in Table 3, the concentration of 600 μg/ml was therefore considered as the minimum dose of G418 for MEC cells selection.

## Transfection and G418 selection of mammal epithelial cells

The MEC cells transfected with pEF-GFP-x*fat1* vector by FuGENE HD Transfection Reagent were selected with G418 for 12 days. As expected, the negative control cells were all dead, and the positive cells could be observed under visible light (Figure 2). However, the expression of eGFP was not evident under fluorescent microscope 48 h following transfection. Subsequently, selection medium was replaced with fresh growth culture media without G418, and the resistant cells formed positive clones and gradually proliferated.

## Identification of positive cells

Total RNA from the monoclonal MEC colonies was extracted using Total RNA Extraction kit (Bioer, China) and the clonal MEC lines examined by RT-PCR amplification. The bright target fragment was detected by electrophoresis on a 1.5% agarose gel in pEF-GFP-x*fat1* transfection group. The GAPDH control confirmed the cDNA quality (189 bp). Both the pEF-GFP-IRES vector negative control and the water group blank control were negative on the gel as shown in Figure 3, suggesting that the transfection was successful and the exogenous gene was integrated into the genome.

## Different expression levels of x*fat1* gene

As expected, the result of Bonferroni's multiple comparison test analysis of real-time RT-PCR indicated that there was no x*fat1* gene mRNA expression in the pEF-GFP-IRES vector control and untransfection control. n*fat1* and y*fat1* showed similar expression level while significantly higher than r*fat1* ($p < 0.05$) (Figure 4).

## Comparison of the catalysis efficiency of three different codon optimization *fat1* gene

Positive transgenic mammary epithelial cells (r*fat1*, n*fat1* and y*fat1*) as sample and pEF-GFP-IRES vector transgenic MEC control cells were used to conduct fatty acid analysis. The results of GC analysis showed that n-3 PUFAs were elevated a lot in n*fat1* group when compared

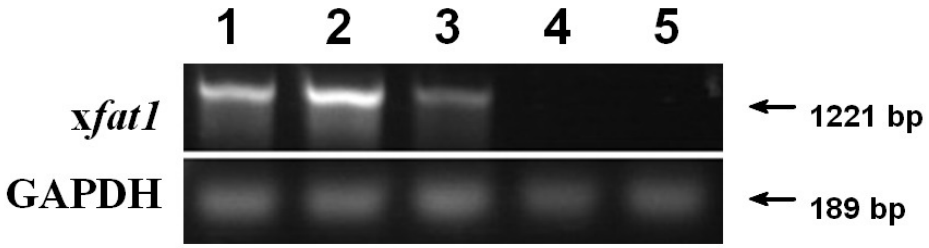


**Figure 3.** Identify positive cells using RT-PCR. 1: r*fat1* positive cells group. 2: n*fat1* positive cells group. 3: r*fat1* positive cells group. 4: pEF-GFP-IRES vector control group. 5: Untransfection control group. pEF-GFP-x*fat1* transfection group showed a 1234, 1221 and 1221 bp fragment, respectively. The GAPDH control confirmed the cDNA quality (189 bp). Both the pEF-GFP-IRES vector group as the negative control and the untransfection group as the blank control were negative result.

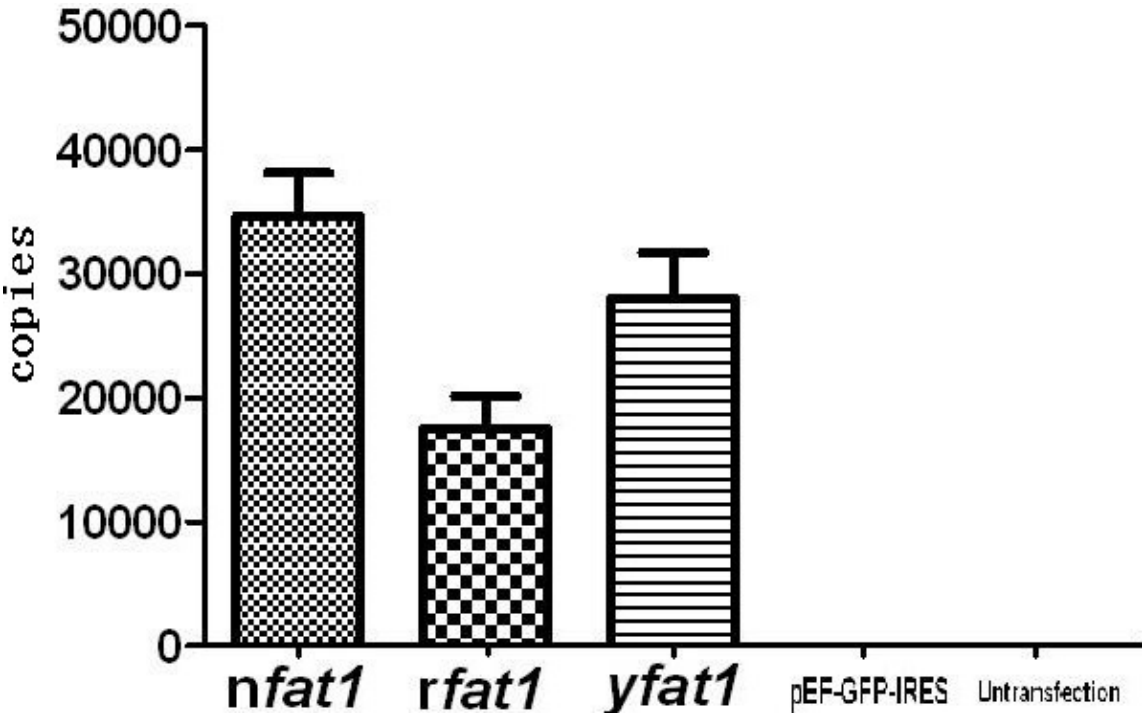


**Figure 4.** The expression of x*fat1* mRNA on MEC cells determined by real-time RT-PCR. Total RNA was extracted from MEC cells and cDNA was prepared using Oligo dT primer. Specificity primers were used to amplify the x*fat1* sequence. The results of Bonferroni's multiple comparison test analysis showed that control groups (pEF-GFP-IRES vector group and untransfection group) had no expression. While n*fat1* and y*fat1* showed analogous express which was significantly higher than r*fat1* ($p < 0.05$).

to the others, and the ratio of n-6/n-3 is 0.7128, which is approximately 2 fold less than control groups. Meanwhile, n-3 PUFAs in r*fat1* and y*fat1* groups also increased. Specifically, the ratio of n-6/n-3 in r*fat1* and y*fat1* is 1.3519 and 1.2061 compared with control MEC cells 1.4353 to 1.4633 as well (Table 4), which are more closed to the ideal ratio 1:1. The specific proportion of total n-3 PUFAs in every group can be seen in Figure 5.

The result of Chi-square test indicated that there are significant differences of the concentration of each Linolenic acid (α-LA, 18:3 n-3) showed a high proportion in n*fat1* and y*fat1* group (49.66 and 40.96%, respectively), which is significantly higher than other groups ($p < 0.05$).

## DISCUSSION

Several n-3 PUFAs between different optimization approaches ($p < 0.05$) (Figure 6). For instance, α-

Table 4. PUFAs composition of total lipids from the transgenic cells and controls.

| Fatty acid | | n*fat1* transgenic cell | r*fat1* transgenic cell | y*fat1* transgenic cell | pEF-GFP-IRES vector control | Untransfection control |
|---|---|---|---|---|---|---|
| n-3 PUFAs | 18:3 n-3 | 1.65440 | 0 | 0.80321 | 0 | 0 |
| | 20:5 n-3 | 0 | 1.52911 | 0 | 0.86193 | 3.23268 |
| | 22:5 n-3 | 0 | 2.20939 | 0 | 1.37282 | 3.93281 |
| | 22:6 n-3 | 0.29083 | 2.00691 | 0.08570 | 1.14540 | 3.10696 |
| Total n-3 PUFAs | | 1.94523 | 5.74541 | 0.88891 | 3.38015 | 10.27245 |
| n-6 PUFAs | 18:2 n-6 | 1.15280 | 3.65069 | 0.7976 | 2.36002 | 7.19989 |
| | 20:4 n-6 | 0.12725 | 4.11629 | 0.16775 | 2.49117 | 7.83222 |
| | 22:4 n-6 | 0.10644 | 0 | 0.10673 | 0 | 0 |
| Total n-6 PUFAs | | 1.38649 | 7.76698 | 1.07208 | 4.85179 | 15.03211 |
| n-6/n-3 | | 0.712764 | 1.3518583 | 1.2060614 | 1.4353771 | 1.4633422 |

optimization *fat1* gene can be expressed functionally in bovine mammary epithelial cells, and their transcription and expression could give MEC cells' different capability of converting n-6 PUFAs to corresponding n-3 PUFAs. It can also lead to an n-6/n-3 ratio balance and a change in HUFAs production. N-3 polyunsaturated fatty acids (n-3 PUFAs) have a specific advantaged effect on biological function and health in mammal, especially in cardiovascular health, cancer and autoimmune diseases (Kris-Etherton et al., 2004; Mozaffarian et al., 2005; Simopoulos, 2002b). Although the ratio of n-6/n-3 PUFAs is also of special importance, a lower ratio of n-6/n-3 is more desirable in reducing the risk of many of the chronic diseases of high prevalence (Simopoulos, 2002a).

There are different codon usage biases in different species, which can influence the expression efficiency of exogenous gene in host tissues and cells. In order to raise the transcription and expression level of specific protein, we could replace the rarity codon sequence via choosing the optimality codon sequence, optimizing and synthesizing CDS into preferences ones. Therefore, the research of codon usage bias plays an important role in increasing protein expression in terms of genetic engineering. Although the expression level of exogenous gene influenced by DNA modification, protein localization etc., the difference of degenerate codon preferences is the key factor. The *C. elegans* is eukaryote, but its codon characteristic is similar with prokaryote. So codon optimization is integrant in this experiment.

The optimization of n*fat1* was based on the codon usage frequency preference of bovine milk protein (casein), including α-LB, LGB, CSN1S1 and CSN2. The r*fat1* gene was based on bovine muscle protein, including myofibrillar proteins, sarcoplasmic proteins and stroma proteins, of which the myofibrillar proteins account for approximately 60% and y*fat1* was mixtype. After optimization, the Frank Wright Nc Value of each x*fat1* showed a significant decrease. The RNA secondary structure analysis showed that the structural stabilization of mRNA is favorable, and the minimum energy reduced distinctly compared with the original *fat1* gene. All the aforementioned is beneficial to raise the expression efficiency (Gao et al., 2003).

The different ways of optimization of *fat1* gene lead to different transcription level in MEC cells. It can be seen clearly that n*fat1* (casein type) shows a significantly high transcription in MEC cells, meanwhile, r*fat1* (muscle type) shows the lowest transcription level, as well as y*fat1* (mixtype) shows intermediate. The specific mechanism is not clear yet, but perhaps it is related to the regulation mechanism of different type of cells. In the pathway of lipometabolism in mammal, PUFAs can be catalyzed by Δ5/Δ6 desaturase and elongase, but n-3 and n-6 PUFAs cannot by mutual transformation. The Δ15 desaturase from *fat1* gene can catalyze almost every type of n-6 PUFAs into n-3 PUFAs in high efficiency, in order to supply the lack of n-3 PUFAs ingestion from food. Meanwhile, the high transcription level of n*fat1* can produce more Δ15 desaturase in MEC, because different level of desaturase compete nearly as many substrate, the catalyst efficiency shows significant difference (Figures 5 and 6).

However, the efficiency of IRES promoter is invalid, it can drive both front and back open reading frame (ORF) in theory, but the distance between IRES and eGFP is unsuitable. Therefore, the expression of eGFP was not evident as fluorescent cells. The optimization of IRES-GFP is under way.

In terms of tissue specific expression, we desired to design a model of muscle and milk expression, in order to produce *fat1* transgenic cattle which contain high proportion of n-3 PUFAs in meat and milk. Ubiquitous

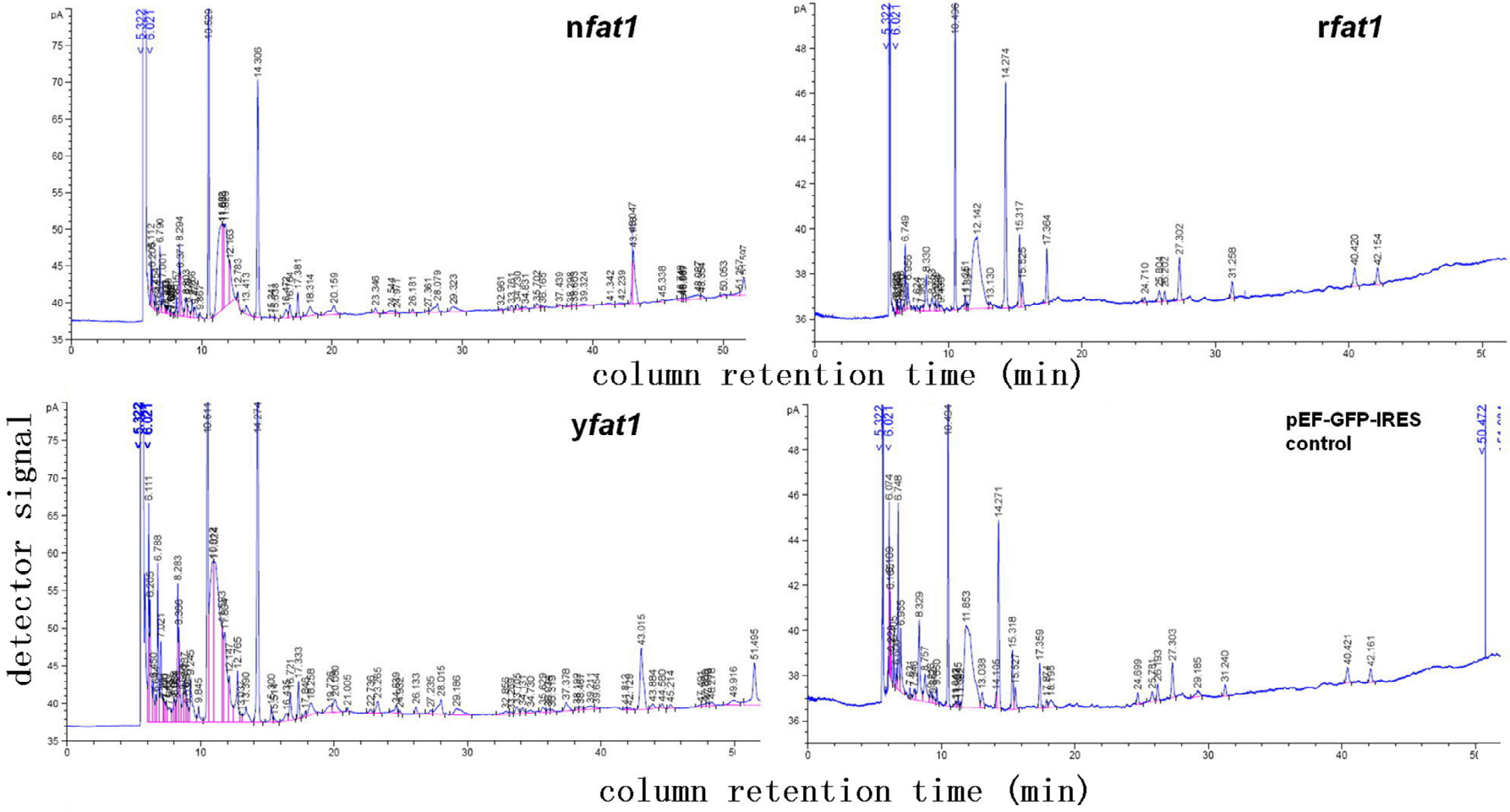


**Figure 5.** Gas chromatograph traces showing fatty acid profiles of total cellular lipids extracted from the positive and control MEC cells. MEC cells were infected with pEF-GFP-x*fat1* and the control groups infected with pEF-GFP-IRES. The results of gas chromatograph showing the catalyst efficiency of n*fat1* group was significantly higher than other groups in terms of total n-3 and n-6 PUFAs

expression was avoided since the possibility of increasing embryonic death rate. It appeared that using a specific promoter did not achieve better results. Animal breast is predominantly composed of MEC, so it was considered an ideal tissue for the production of n-3 PUFAs by transferring the n-3 fatty acid desaturase gene. We proved that different ways of codon approach can play a role in specificity express proteins in MEC. However, our work demonstrated that the composition of n-3 PUFAs changed a lot in different optimized approach. Therefore, the cause of this phenomenon needs to be explored in the future.

Thus, our studies indicated that milk protein optimization approach (n*fat1*) can produce high n-

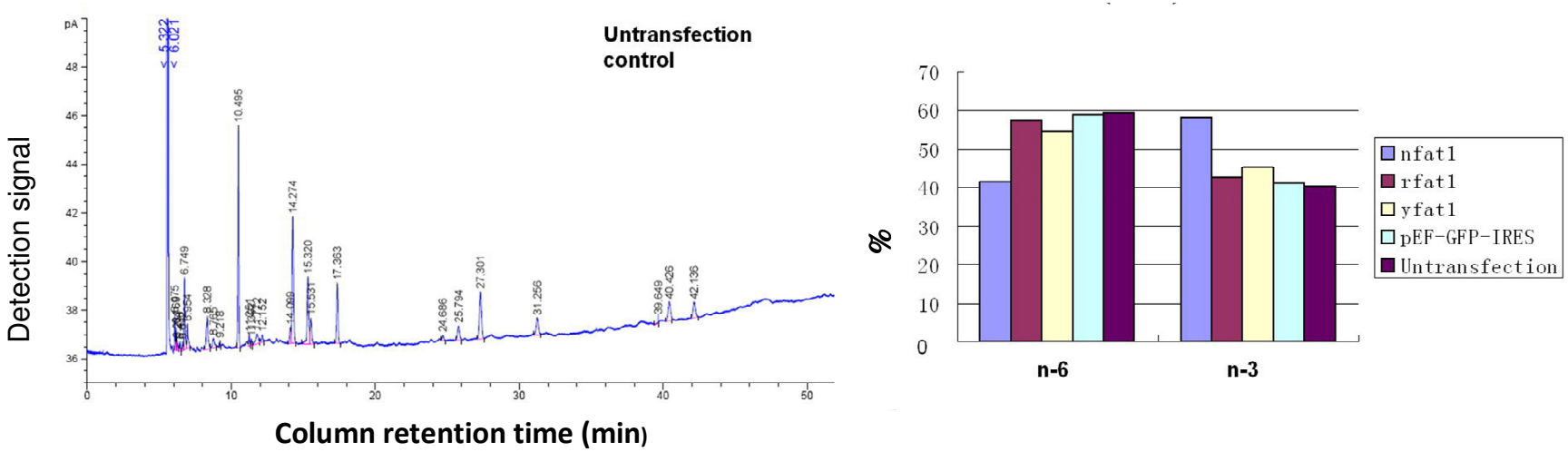


**Figure 5.** Gas chromatograph traces showing fatty acid profiles of total cellular lipids extracted from the positive and control MEC cells. MEC cells were infected with pEF-GFP-x*fat1* and the control groups infected with pEF-GFP-IRES. The results of gas chromatograph showing the catalyst efficiency of n*fat1* group was significantly higher than other groups in terms of total n-3 and n-6 PUFAs.

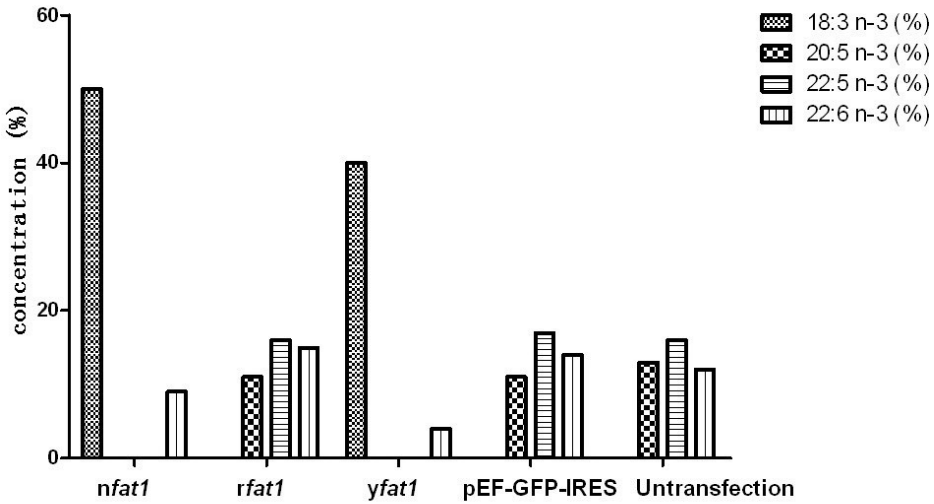


**Figure 6.** The Chi-square test results of concentration of each type of n-3 PUFAs between different optimization approaches. Values are means ± SD of all experiments and expressed as a percentage of control ($p < 0.05$).

3 PUFAs in MEC, which probably become an efficient and economical approach to produce milk enriched in n-3 PUFAs transgenic domestic animals.

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### REFERENCES

Comeron JM, Aguade M (1998). An evaluation of measures of synonymous codon usage bias. J. Mol. Evol. 47: 268-274.

Enser M, Hallett K, Hewitt B, Fursey GAJ, Wood JD (1996). Fatty acid content and composition of english beef, lamb and pork at retail. Meat Science, 42: 443-456.

Enser M, Hallett KG, Hewitt B, Fursey GAJ, Wood JD, Harrington G (1998). Fatty acid content and composition of UK beef and lamb muscle in relation to production system and implications for human nutrition. . Meat Science, 49: 329-341.

Ermolaeva MD (2001). Synonymous codon usage in bacteria. Curr. Issues Mol. Biol. 3: 91-97.

Gao X, Yo P, Keith A, Ragan TJ, Harris TK (2003). Thermodynamically balanced inside-out (TBIO) PCR-based gene synthesis: a novel method of primer design for high-fidelity assembly of longer gene sequences. Nucleic Acids Res. 31: e143.

Grantham R, Gautier C, Gouy M (1980a). Codon frequencies in 119 individual genes confirm consistent choices of degenerate bases according to genome type. Nucleic Acids Res. 8: 1893-1912.

Grantham R, Gautier C, Gouy M, Mercier R, Pave A (1980b). Codon catalog usage and the genome hypothesis. Nucleic Acids Res. 8: r49-r62.

Kang JX, Wang J (2005). A simplified method for analysis of polyunsaturated fatty acids. BMC Biochem. 6: 5.

Kris-Etherton PM, Hecker KD, Binkoski AE (2004). Polyunsaturated fatty acids and cardiovascular health. Nutri. Rev. 62: 414-426.

Lai L, Kang JX, Li R, Wang J, Witt WT, Yong HY, Hao Y, Wax DM, Murphy CN, Rieke A, Samuel M, Prather RS, Dai Y (2006). Generation of cloned transgenic pigs rich in omega-3 fatty acids. Nat. Biotechnol. 24: 435-436.

Lopez-Huertas E (2010). Health effects of oleic acid and long chain omega-3 fatty acids (EPA and DHA) enriched milks. A review of intervention studies. Pharmacol. Res. 61: 200-207.

Lu Y, Nie D, Witt WT, Chen Q, Shen M, Xie H, Lai L, Dai Y, Zhang J (2008). Expression of the fat-1 gene diminishes prostate cancer growth in vivo through enhancing apoptosis and inhibiting GSK-3 beta phosphorylation. Mol. Cancer Ther. 7: 3203-3211.

Marra MA, Jones SJ, Astell CR, Holt RA, Brooks-Wilson A, Butterfield YS, Khattra J, (2003). The Genome sequence of the SARS-associated coronavirus. Science, 300: 1399-1404.

Mozaffarian D, Ascherio A, Hu FB, Stampfer MJ, Willett WC, Siscovick DS, and Rimm EB (2005). Interplay between different polyunsaturated fatty acids and risk of coronary heart disease in men. Circulation. 111: 157-164.

Olivotto I, Di Stefano M, Rosetti S, Cossignani L, Pugnaloni A, Giantomassi F, Carnevali O (2011). Live prey enrichment, with particular emphasis on HUFAs, as limiting factor in false percula clownfish (Amphiprion ocellaris, Pomacentridae) larval development and metamorphosis: molecular and biochemical implications. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 159: 207-218.

Pereira SL, Huang YS, Bobik EG, Kinney AJ, Stecca KL, Packer JC, Mukerji P (2004). A novel omega3-fatty acid desaturase involved in the biosynthesis of eicosapentaenoic acid. Biochem. J. 378: 665-671.

Sakuradani E, Abe T, Iguchi K,Shimizu S (2005). A novel fungal omega3-desaturase with wide substrate specificity from arachidonic acid-producing Mortierella alpina 1S-4. Appl. Microbiol. Biotechnol. 66: 648-654.

Simopoulos AP (2002a). The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed. Pharmacother. 56: 365-379.

Simopoulos AP (2002b). Omega-3 fatty acids in inflammation and autoimmune diseases. J. Am. Coll. Nutri. 21: 495-505.

Spychalla JP, Kinney AJ, Browse J (1997). Identification of an animal omega-3 fatty acid desaturase by heterologous expression in Arabidopsis. Proc. Natl. Acad. Sci. USA. 94: 1142-1147.

Stothard P (2000). The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques, 28: 1102-1104.

Wright F (1990). The 'effective number of codons' used in a gene. Gene. 87: 23-29.

Zhu G, Chen H, Wu X, Zhou Y, Lu J, Deng J (2008). A modified n-3 fatty acid desaturase gene from Caenorhabditis briggsae produced high proportion of DHA and DPA in transgenic mice. Transgenic Res. 17: 717-725.