Original Paper

The Generation of Transgenic Mice with *Fat1* and *Fad2* Genes that have their own Polyunsaturated Fatty Acid Biosynthetic Pathway

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Key Words

Polyunsaturated fatty acids (PUFAs) • Omega-3 PUFAs • Omega-6 PUFAs • *Fat-1* • *Fad-2* • Transgenic mice

Abstract

Background: Microorganisms and higher plants possess their own omega-3 and omega-6 polyunsaturated fatty acid (PUFAs) biosynthetic pathways. The n-6 fatty acid desaturase gene *fad-2* codes for the n-6 desaturase enzyme that converts oleic acid (OA 18:1 n-9) into linoleic acid (LA 18:2 n-6). The n-3 fatty acid desaturase gene *fat-1* codes for the n-3 desaturase enzyme that converts n-6 PUFAs into n-3 PUFAs. Mammals lack n-3 and n-6 desaturase enzymes; therefore, they must obtain their omega-3 and omega-6 fatty acids by consuming plants or seafood. The beneficial effects of n-3 and n-6 PUFAs on human development and cardiovascular health have been well documented. **Methods:** Here, we generated *fat-1* and *fad-2* transgenic mice by introducing mammal expression vectors containing the *fat-1* and *fad-2* genes via microinjection. **Results:** Seven transgenic mice were obtained that expressed functional n-3 and n-6 desaturase enzymes. Analysis of the fatty acid contents of transgenic mouse livers revealed that n-6 and n-3 PUFA levels were greatly increased in the transgenic mice compared to wild-type mice. The use ratios of n-9 PUFAs (18:1 n-9) and n-6 PUFAs were both greater in the transgenic mice than in the wild-type controls. **Conclusion:** These transgenic mice were capable of producing their own omega-3 and omega-6 fatty acids. They have the same fatty acid metabolic pathways as higher plants and microbes.

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Introduction

Microorganisms and plants have their own omega-3 and omega-6 PUFA biosynthetic pathways [1]. The n-6 desaturase enzyme, coded by the *fad-2* gene, introduces a double bond at position $\Delta 12$ to convert oleic acid (OA 18:1 n-9) into linoleic acid (LA 18:2 n-6). The n-3 desaturase enzyme, coded by the *fat-1* gene, introduces a double bond at position $\Delta 15$ to convert linoleic acid (LA 18:2 n-6) to α -linolenic acid (ALA 18:3 n-3). The n-3 desaturase enzymes have broad omega-6 PUFA substrate specificity, allowing them to convert γ -linolenic acid, di-homo- γ -linolenic acid, and arachidonic acid into eicosapentaenoic acid (EPA 20:5 n-3), docosapentaenoic acid (DPA 22:5 n-3), and docosahexaenoic acid (DHA 22:6 n-3), respectively. *C. elegans* is a good model in this respect because it is an animal that possesses an n-3 desaturase activity. Their n-3 desaturase enzymes elaborate a wide range of n-3 PUFAs including arachidonic and eicosapentaenoic acids [2-4]. The n-6 PUFAs and n-3 PUFAs are typified by linoleic acid (LA) and α -linolenic acid (ALA), respectively. Both ALA and LA are metabolised to longer-chain PUFAs, primarily in the liver.

Omega-3 and omega-6 PUFA biosynthetic pathways do not occur in mammals; therefore, mammals must obtain omega-3 and omega-6 PUFAs from their diets [5]. Linoleic acid (LA 18:2 n-6) and α -linolenic acid (ALA 18:3 n-3) are essential fatty acids that are required for normal growth in mammals. These PUFAs are integral components of cell membranes. They also play important roles in determining the structures of the eicosanoids that regulate the release of hypothalamic and pituitary hormones [6]. Furthermore, highly unsaturated PUFAs, such as arachidonic acid, docosatetraenoic acid, and docosahexaenoic acid, are found in high concentrations in the structural lipids of the central nervous system and are essential in infant nutrition [7, 8]. *C. elegans* mutants lacking fatty acid desaturase genes have PUFA deficiencies that cause growth and neurological defects, such as slow growth, abnormal body shape, sluggish movement, cuticle defects, and reduced brood size [9].

Saeki et al. generated transgenic pigs that carried the *fad-2* gene for n-6 fatty acid desaturase from spinach. Adipocytes differentiated *in vitro* from the transgenic pigs had higher LA levels [10]. Kang et al. generated healthy humanised transgenic mice expressing the *fat-1* gene (*hfat-1*) with the n-3 fatty acid desaturase from *C. elegans*. These transgenic mice produced significantly more n-3 fatty acids and had a reduced n-6/n-3 ratio compared with wild-type mice [11]. The transgenic mice also had significantly elevated n-3 fatty acid levels in their milk. Neonatal mice fed with milk from transgenic mothers had increased levels of docosahexaenoic acid (DHA) in their brain tissue [12, 13]. Lai et al. reported that expression of *hfat-1*, encoding the n-3 fatty acid desaturase from *C. elegans*, significantly increased the n-3 fatty acid content and sharply decreased the ratio of n-6/n-3 fatty acids in transgenic pigs [14]. Wu et al. generated *mfat-1* transgenic cattle that had significantly reduced n-6/n-3 ratios in both their tissue and milk [15]. Endogenous production of n-3 and n-6 fatty acids in mammalian cells has also been described [16]. Other reports have shown that fatty acid desaturase genes isolated from higher plants, such as maize [17], spinach [10], and scarlet weeds [18], function well in mammals after being introduced into animal cells.

However, at present, there are no mammal systems that possess both omega-3 and omega-6 PUFA biosynthetic pathways. Here, we report the production of transgenic mice that carry cDNA for *fad-2* from spinach and *fat-1* from *C. elegans*. These genes were introduced by microinjection of plasmids into fertilised mice eggs. We detected functional expression of the *fad-2* and *fat-1* genes in these transgenic mice and analysed the composition of PUFAs in their livers.

Materials and Methods

Reagents and animals

All reagents were purchased from Sigma unless otherwise noted. Kunming (KM) mice and ICR mice were purchased from the Center of Experimental Animals of Jilin University and Changchun Hi-Tech

Fig. 1. Structure of the expression vector pIRES-FAT1-FAD2. The fat-1 gene was inserted into the pIRES vector using the restriction enzymes *XhoI* and *EcoRI*, the vector was then named pIRES-FAT1. Afterwards, the fad2 gene and vector pIRES-FAT1 were digested by restriction enzymes *Sall* and *NotI*, re-ligate to construct the pIRES-FAT1-FAD2 vector.

Laboratory Animal Research Center, respectively. The daily diet fed for the mice is a mixture of corn, fishmeal, beans, grain, flour, calcium perphosphate and powder (Nutrition Facts: Protein 18%, Fat 4%, Fiber less than 5%, Powder less than 8%, Calcium 1-1.8%, Phosphorus 0.6-1.2%, Water less than 10%). Animals were housed under controlled light cycle (14 h light/10 h dark) conditions. Animal treatments complied with a protocol approved by the Jilin University Institutional Animal Care and Use Committee.

Construction of the fat-1 and fad-2 expression vector

The coding region of the *fat-1* gene from *C. elegans* (GenBank: NM_001028389) was optimised for mammalian cell expression and introduced into the pIRES expression vector at *XhoI* and *EcoRI* restriction sites. The coding region of the *fad-2* gene from *S. oleracea* (GenBank: AB094415.1) was obtained by RT-PCR using the following primers: upstream, 5'-ACG CGT CGA CCA CAC TTC GGC CTC TCC TTC TCC TT-3'; and downstream, 5'-AAA AGC GGC CGC AAT TCA CAA ATA ATT GTG TCC CAT-3'. After sequencing, the *fad-2* fragment was introduced into the *fat-1* expression pIRES-fat1 vector at *Sall* and *NotI* restriction sites. The mammalian expression vector containing the *fat-1* and *fad-2* genes was named pIRES-FAT1-FAD2 (Fig. 1).

Generation of transgenic mice

Six-week-old female ICR mice were superovulated by intraperitoneal injection of 10 IU (international units) of pregnant mare's serum gonadotropin (PMSG, Ningbo, China), followed by intraperitoneal injection of 10 IU of human chorionic gonadotropin (hCG, Ningbo, China) at intervals of 48 h. To generate zygotes, ICR mice were mated with KM (Kunming) male mice at a 1:1 ratio after hCG injection. Females with vaginal plugs were used for zygote preparation. Plugged females were sacrificed at day 0.5, and the zygotes were collected from their oviducts and denuded from the cumulus cells by treatment with 0.2% hyaluronidase. For pronuclear microinjection, the pIRES-fat1-fad2 expression vector was excised by digestion with *AhdI*. The purified transgene was diluted in TE buffer (2 to 3 ng/mL) and was microinjected into the male pronucleus of zygotes under an inverted microscope (Nikon, Japan). The zygotes were then cultured in KSOM (Millipore) at 37°C in a 5% CO₂, 95% air environment overnight. Embryos at the two-cell stage of development that survived the microinjection were transferred into the oviducts of 0.5-dpc pseudo pregnant female ICR mice.

Transgene detection by RT-PCR

RT-PCR was carried out to detect the expression of the *fad2* and *fat1* genes in the off spring of the transgenic founders, including both transgenic and wild-type littermates. The total RNA was extracted from tail muscle tissue using the TRIzol Reagent according to the manufacturer's instructions. RNA samples were treated with DNase prior to RT-PCR. Purified RNA (500 ng) was used for first-strand cDNA synthesis. Reverse transcription was performed using oligo-dT primers at 42°C for 1 h and then at 90°C for 5 min. The resulting cDNA was used for PCR with primers specific for *fad2*: upstream, 5'-ACC CAT CCT CTC CAA

TCT ACT C-3'; and downstream, 5'-GTC TTC ATC AGC CTC CAC ATA C-3'. Amplification of the *GAPDH* gene was used as a control using the following primers: upstream, 5'-ACG TGC CGC CTG GAG AAA CC-3'; and downstream, 5'-GGC CAT GAG GTC CAC CAC CCT G -3'. PCR was carried out by heating at 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. RNA samples from the muscle tissue of wild-type mice were used as negative controls. Plasmids containing *fad2* fragments were used as positive controls.

Southern blotting analysis

Genomic DNA (20 µg) from the tail tissue of transgenic and non-transgenic mice was digested using *HindIII*. The digested products were electrophoresed on a 0.8% agarose gel using TAE buffer and were transferred onto Hybond-N membranes (Invitrogen). Southern blotting was performed using a 479-bp specific hybridisation probe that was labelled with digoxigenin (DIG) using a PCR DIG Probe Synthesis kit (Roche Applied Sciences, Mannheim, Germany). The PCR parameters were as follows: 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final extension step of 72°C for 10 min. The hybridisation probe primer sequences were as follows: upstream, 5' -ACC CAT CCT CTC CAA TCT ACT C-3'; and downstream: 5' -GTC TTC ATC AGC CTC CAC ATA C-3'. Hybridisation and immunological detection were performed according to the instructions provided with the DIG High Prime DNA Labeling and Detection Starter Kit (Roche Applied Sciences, Mannheim, Germany). The positive hybridisation signal was a 3-kb fragment. Genomic DNA from non-transgenic mice was used as a negative control. The pIRES-fat1-fad2 plasmid DNA (8.9 kb) was used as a positive control.

Integration site analysis in transgenic mice by thermal asymmetric interlaced TAIL-PCR

TAIL-PCR was used to define both the 5' and 3' chromosomal boundaries of the 8.9-kb transgene of the transgenic mice using gene specific primers (GSP) and six arbitrary degenerate primers (AD).

A series of 3 PCR reactions (designated primary, secondary, and tertiary TAIL) were performed. The AD primers were as follows: NGTCGASWGANAWGAA, TGWGNAGSANCASAGA, AGWGNAGWANCAWAGG, STTGNTASTNCTNTGC, NTCGASTWTSGWGTT and WGTGNAGWANCANAGA. The GSPs were 21–24 nucleotides long, with melting temperatures of approximately 64°C and GC contents of 50–60%. The GSPs were as follows: ACCGTACACGCCTACGCCCCATTT, AGGCGCAGACGCAACAGCAAATGC, and GCCAGCCAAGGGTTAGCGTCACAA. The first TAIL-PCR was performed as follows: 94°C (5 min), 10 cycles of 94°C (10 s), 64°C (30 s), 72°C (3 min), 1 cycle of 94°C (10 s), 25°C (3 min), 72°C (2.5 min), 15 cycles of 94°C (10 s), 64°C (3 min), 72°C (2.5 min), 94°C (10 s), 64°C (3 min), 72°C (2.5 min), 94°C (10 s), 44°C (1 min), and 72°C (2.5 min). Secondary TAIL-PCR was performed as follows: 94°C (5 min), followed by 12 cycles of 94°C (10 s), 64°C (3 min), 72°C (2.5 min), 94°C (10 s), 64°C (3 min), 72°C (2.5 min), 94°C (10 s), 44°C (1 min), and 72°C (2.5 min). Tertiary TAIL-PCR was performed as follows: 94°C (5 min), followed by 20 cycles of 94°C (10 s), 44°C (1 min), and 72°C (2.5 min).

PUFA analysis

Male transgenic mice were mated with female C57BL/6 mice to yield non-transgenic and transgenic mice. Six-week-old female offspring were used for PUFA analysis by gas chromatography, as previously described [19]. Fresh mouse liver tissue was homogenised by grinding it in liquid nitrogen, and an aliquot of the tissue homogenate in a glass methylation tube was mixed with 1.5 mL of hexane and 1.5 mL of 14% BF₃/MeOH reagent (Sigma). After blanketing in nitrogen, the mixture was heated at 100°C for 1 h and then cooled down to room temperature; methyl esters were extracted in the hexane phase following the addition of 1.5 mL of H₂O. The samples were centrifuged at 3000 r/min for 1 min, and then, the upper hexane layer was removed. Fatty acid methyl esters were analysed by gas chromatography using a fully automated 7890 Network GC System (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame-ionisation detector. Chromatography was performed with an Agilent J&W fused-silica capillary column (DB-23; 60 m, 0.25-µm film thickness, 0.25-mm i.d.; Agilent). The injector and detector ports were set at 250°C. FAMES were eluted using a program held the temperature at 180°C for 6 min and increased by 6.5°C/min to reach 205°C for 3 min, before increasing again at 3°C/min to reach 240°C for 3 min. The carrier gas was helium at a constant pressure of 230 kPa. Peaks were identified by comparison with fatty acid standards. The area percentages for all resolved peaks were analysed using GC ChemStation Software (Agilent Technologies).

Fig. 2. The selection of transgenic founders by genomic PCR. Left: Detection of *fat-1* gene 450bp. M: Marker 2000; 01–05 samples from F0 mice; –: negative control samples from non-transgenic mice; +: positive control samples from expression vector. Right: Detection of *fad-2* gene 479bp. M: Marker2000; 01–05 samples from F0 mice; –: negative control samples from non-transgenic mice; +: positive control samples from expression vector plasmid; H: ddH₂O.

Fig. 3. The detection of transgenic mice by RT-PCR and Southern blotting. RT-PCR analysis the *fad-2* cDNA from tail samples of the transgenic mice. The PCR product for *fad-2* cDNA is 479bp. GAPDH is the control. The PCR product is 252bp. Southern blotting analysis the transgenic mice. The positive hybridization signal is 3 kb. Positive control is the pIRES-fat1-fad2 vector. Negative control is the genomic DNA of wild type mouse.

Mouse	Number of the integration sites	Localization of the integration sites
F0	4	chromosome 2, chromosome 7, chromosome 3, chromosome 10

Table 1. Determination of integration sites and zygosity by Tail-PCR

Results

Generation of transgenic mice that express both fad-2 and fat-1 genes

The linear transgenic cassettes containing both *fad-2* and *fat-1* genes were microinjected into the male pronuclei of fertilised eggs that were isolated from super ovulated female mice. Two recipients transferred with a total of 40 embryos became pregnant and gave birth to 5 (12.5%) offspring. PCR analysis of DNA samples from the offspring showed transgenes in one male mouse (Fig. 2). At 6 weeks old, this mouse with transcriptionally active *fat-1* and *fad-2* was mated with wild-type female mice, producing 6 more offspring. These offspring were all positive by RT-PCR (Fig. 3).

RT-PCR and Southern blotting confirmed the presence of the transgene

RT-PCR analysis of the samples showed the presence of the transgenes in one (F0) and six (F1) mice. To confirm the integration of the transgene, Southern blotting analysis (Fig. 3) was carried out using a *fad2* DNA probe designed to hybridise with a 1338bp fragment to demonstrate that all RT-PCR positive mice integrated the transgenes. All 7 founders were fertile and developed into normal adults.

Fig. 4. Functional expression of the *fad2* gene and *fat1* gene in the F0 transgenic mice.

Table 2. Liver PUFA compositions of F1 transgenic mice and the controls

PUFAs	Wildtype mice (n=4)	Transgenic mice (n=6)
18:2 n-6	10.01±0.75	16.02±2.15
18:3 n-6	0.42±0.05	0.489±0.0612
20:4 n-6	0.09±0.001	0.12±0.0198
22:5 n-6	0.75±0.08	1.2±0.159
18:3 n-3	0.315±0.0295	0.259±0.031
20:5 n-3	0.216±0.03	0.277±0.035
22:5 n-3	0.18±0.01	0.545±0.0679
22:6 n-3	2.39±0.289	4.1±0.512
N-6 PUFAs	10.01±0.881	17.829±2.39
N-3 PUFAs	3.101±0.3585	5.181±0.6459

Tail-PCR to determine integration sites and zygosity
Integration site analysis of the F0 transgenic mouse was performed using TAIL-PCR. There were 4 integration sites found across chromosomes 2, 3, 7, and 10 (Table 1).

	Transgene copy number	Total n-6 PUFAs content	Total n-3 PUFAs content
F1-001	4	18.93	5.23
F1-002	6	15.79	4.87
F1-006	12	16.84	5.78

Table 3. Transgene copy number and concentration of PUFAs in F1 transgenic mice

Liver PUFA composition of the transgenic mice

To assess the function of the fat1 and fad2 enzymes in the F0 transgenic mouse, the PUFA content in the liver tissue was analysed. As shown in Figure 4, both n-6 and n-3 PUFAs were significantly increased relative to the wild-type values. These data indicate that the fat1 and fad2 enzymes function in the F0 transgenic mice. For the detailed analysis of fat1 and fad2 enzyme function, F1 heterozygous transgenic mice and non-transgenic mice were used for PUFA measurement. As shown in Table 2, the concentration of oleic acid (18:1 n-9) was significantly reduced in transgenic mouse liver (3.04 ± 1.08) compared with the non-transgenic mouse liver (19.2 ± 1.5), although the concentrations of linoleic acid (LA 18:2 n-6) were not altered ($P > 0.05$) between the transgenic liver (16.02 ± 2.15) and the non-transgenic liver (10.01 ± 0.75) (Table 2). Our results indicate that *fad-2* is functionally expressed in a complex mammalian system and that *fad-2* expression in transgenic mice actively converts oleic acid into LA.

Due to a lack of fat-1 desaturase, mammals are unable to synthesise n-3 fatty acids. Therefore, they must obtain them from their diet. The n-3 fatty acids, including 18:3, 20:5, 22:5, and 22:6, are derived from dietary ALA. *Fad-2* and *fat-1* expression in transgenic mice promotes *de novo* biosynthesis of LA and n-3 PUFAs. Therefore, we analysed the n-3 PUFAs, including ALA (18:3 n-3), EPA (20:5 n-3), DPA (22:5 n-3), and DHA (22:6 n-3). As expected, the n-3 fatty acid levels in liver tissue from transgenic mice were significantly higher than those in non-transgenic mice. Liver tissue from transgenic mice contained 67.08% more n-3 PUFAs than liver tissue from non-transgenic mice. In addition, the levels of EPA, DPA, and DHA from the transgenic liver tissues increased by 28.24%, 202.7%, and 71.55%, respectively.

Transgene copy number and concentration of PUFAs in F1 transgenic mice

To better understand the association between genotype and phenotype, we investigated the relationship between the transgene copy number and the PUFA contents of F1 transgenic mice. As shown in Table 3, there was no direct association between the copy number and the amount of total n-6 and n-3 PUFAs. Different expression levels may be attributed to complex factors, including the genomic context of the gene [20], the transgene methylation status [21], and the transgene copy number.

Discussion

As we know, the fad2 and fat1 desaturase enzymes are the two of the most important fatty acid desaturases. Due to a lack of both of these enzymes, mammals are unable to synthesise omega-6 fatty acids and omega-3 fatty acids; therefore, they must rely obtaining these fatty acids from dietary sources.

Previous studies about PUFAs mainly focused on changing the ratio between n-3 fatty acids and n-6 fatty acids in diets to improve health [1, 22]. Such studies might yield conflicting results due to the inevitable variance in the dietary composition and eating habits of humans and animals [23]. In our *fad2* and *fat1* transgenic mice, the fad2 desaturase enzymes promoted *de novo* biosynthesis of n-6 PUFAs, and the fat1 desaturase enzymes converted the n-6 PUFAs into n-3 PUFAs. We analysed the oleic acids, LA and ALA, as well as

downstream *n*-3 fatty acids, including EPA, DPA, and DHA. Livers from transgenic mice had higher levels of both *n*-6 and *n*-3 PUFAs than controls, suggesting that our transgenic mice can synthesise omega-3 and omega-6 PUFAs. These mice have the same fatty acid metabolic pathways as higher plants and microbes.

In general, vegetable oils, beef, and chicken contain high levels of *n*-6 fatty acids, whereas fish oils contain high levels of *n*-3 fatty acids. The modulation of fatty acid content by inducing the expression of foreign desaturase genes in transgenic animals might reveal the effect that fatty acids have on the body [16, 24]. Transgenic animal models expressing plant *fad*-2 genes have been generated [10, 25]. Furthermore, *n*-6 fatty acids have been indicated in several diseases, such as hyperinsulinism, arteriosclerosis, and cancer [26-29]. Transgenic animal models expressing the *C. elegans fat-1* gene have also been generated [11, 14, 30]. The *fat-1* transgenic mice have now been widely used to study the effects of fatty acids on various health conditions [31-35]. PUFAs serve as signalling molecules and as precursors for other signalling molecules, such as prostaglandins, leukotrienes, and thromboxanes, and can exert a wide range of biological effects. Deficiencies in *n*-6 or *n*-3 PUFAs may cause different symptoms in mammals. A lack of *n*-3 PUFAs results in nervous system disease, whereas a lack of *n*-6 PUFAs results in non-neuronal abnormalities, including reduced growth, reproductive failure, skin lesions, fatty liver, and polydipsia [36]. Due to the importance of both *n*-6 and *n*-3 PUFAs, monitoring both the ectogenic balance of *n*-3/*n*-6 ratios and the endogenic synthetic amounts of *n*-6 and *n*-3 could be important.

Our transgenic mice, which express both *fad2* and *fat1* desaturase enzymes, have their own PUFA biosynthetic pathways. In the future, we will continue to work on improving the endogenic balance of *n*-3 and *n*-6 PUFAs. In addition to the *fad2* transgenic mice created by Chen et al. [25] and the *fat1* transgenic mice created by Kang et al [11], the *fad2* and *fat1* transgenic mice we produced may represent an opportunity to further study the various diseases caused by deficiencies in *n*-6 and *n*-3 PUFAs. Furthermore, the *fad2* and *fat1* transgenic animals may serve as references for the commercial production of transgenic animals.

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