Cite this article: Song L, Yang L, Wang J, Liu X, Bai L, Di A, Li G. 2019 Generation of Fad2 and Fad3 transgenic mice that produce n-6 and n-3 polyunsaturated fatty acids. Open Biol. 9: 190140.
http://dx.doi.org/10.1098/rsob.190140

Received: 26 June 2019
Accepted: 20 September 2019

Subject Area: biotechnology/cellular biology/molecular biology

Keywords: polyunsaturated fatty acids (PUFAs), n-3/6 PUFAs, fatty acid desaturase (Fad), Fad2/3, transgenic mice

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.4681277.

1. Introduction
Polyunsaturated fatty acids (PUFAs) consist of more than two double bonds, and are required for mammalian and human health [1,2]. There are two main categories of PUFAs: n-6 PUFAs (also known as omega-6, ω-6) and n-3 PUFAs (or ω-3) [3]. The n-6 PUFAs mainly include linoleic acid (LA; 18 : 2, n-6), γ-linolenic acid (γ-LA; 18 : 3, n-6) and arachidonic acid (AA; 20 : 4, n-6). The n-3 PUFAs mainly include α-linolenic acid (ALA; 18 : 3, n-3), eicosapentaenoic acid (EPA; 20 : 5, n-3) and docosahexaenoic acid (DHA; 22 : 6, n-3) [1,4].

In a human newborn study, PUFAs are required to maintain normal retinal and brain development [5]. For children and adults, PUFAs can reduce the risk of some diseases such as vascular diseases, arthritis, cancer and neurological diseases [6–9]. Plants and microorganisms can synthesize PUFAs by themselves because they have their own desaturases [3,10]. However, mammals are unable to synthesize n-6 and n-3 PUFAs [11], because they lack delta (Δ)-12 and Δ-15 desaturases. Therefore, mammals and humans must consume the necessary n-6 and n-3 PUFAs in their diet.

Desaturation is an important biochemical process in fatty acid biosynthesis. Previous studies have shown that fatty acid desaturase (Fad) plays an important role in fatty acid desaturation [12,13]. The Fad enzymes convert saturated fatty acids with a single bond between two carbon atoms to unsaturated fatty acids with a double bond at a specific location in the fatty acyl chain [14,15]. The Fad2 enzyme introduces a double bond at the Δ12 position in the monounsaturated fatty acid oleic acid (OA; 18 : 1, n-9) to form n-6 LA, and the Fad3 enzyme...
Figure 1. Generation of transgenic mice that express the Fad3 gene. (a) Schematic representation of pCMV–Fad3 plasmid. (b) Summary of transgenic mice that carried the Fad3 gene. (c) Identification of Fad3 transgenic mice by PCR. (d) Validation of Fad3 transgene expression in seven major tissues by RT-PCR. 

2. Material and methods

2.1. Animals and chemicals

The CD1, Kun-Ming (KM) and BDF1 (C57BL/6N × DBA/2) mice were purchased from the Experimental Animal Research Center (Inner Mongolia University). Mice were housed in a specific pathogen-free room with an appropriate temperature (22–24°C) and light–dark cycle (light 8.30–20.30), and were fed with a regular diet. All chemicals used in this study were purchased from Sigma (St Louis, MO, USA), unless otherwise indicated.

2.2. Expression vector construction of the Fad2/3 gene

The Fad2 and Fad3 genes used in this study were PCR-amplified from the cDNA of Spinacia oleracea and Linum usitatissimum (also known as common flax or linseed), respectively. Total RNA was extracted from S. oleracea and L. usitatissimum using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The complete open reading frames and partial up- and downstream noncoding regions of the Fad2/3 genes from cDNA were amplified using Phanta Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China). The PCR products were cloned into the pMD19-T vector (Takara, Kusatsu, Japan) and sequenced according to the manufacturer’s instructions. After sequencing, the Fad3 cDNA was introduced into the pIRES expression vector at the XhoI and MluI restriction sites, and the vector was named pCMV–Fad3 (figure 1a). Then, the Fad2 gene was introduced into the pCMV–Fad3 vector at the SalI and NotI restriction sites, and this was named pCMV–Fad2–Fad3 (figure 3a). The primer information was presented in electronic supplementary material, table S5.
2.3. Generation of transgenic mice

The pronuclear microinjection was carried out as described previously [28,29]. Briefly, 6–8-week-old BDF1 female mice were superovulated through intraperitoneal (i.p.) injection of pregnant mare serum gonadotropin (10 IU; Sansheng, Ningbo, China) and human chorionic gonadotropin (hCG, 10 IU; Sansheng), 48 h apart. After the hCG i.p. injection, the female mice mated with male BDF1 mice in a 1 : 1 ratio in single cages overnight. To obtain pseudo-pregnant surrogate mice, two CD1/KM females were placed together with one vasectomized male overnight. The next morning, successful mating was confirmed by the presence of vaginal plugs. The plugged female mice were sacrificed through cervical dislocation, and zygotes were collected from the oviducts using a stereoscopic microscope (Nikon, Tokyo, Japan). To prepare DNA for microinjection, pCMV Fad3 expression vectors were linearized and purified using the Plasmid Plus Kit (Qiagen) in accordance with the manufacturer’s protocol. After purification, the DNA was diluted to 3 ng µl⁻¹ in modified TE buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA). The injection needle was filled with 1 µl of the DNA suspension. The linearized vector was injected into the well-recognized pronuclei. After injection, zygotes were maintained at room temperature for 30 min and then moved into the incubator. The injected zygotes were transferred into pseudo-pregnant female mice (approx. 30 zygotes per mouse) after a 2 h recovery culture in KSOM-AA medium. After 19–21 days, the mice pups were delivered naturally. For founder identification, the tail tips (approx. 1 cm) were subjected to standard DNA extraction procedures. The amplified DNA fragments were subjected to TA cloning and sequencing. The founder (F0) mice were crossed with their littermates to produce F1 mice. The primer information was presented in electronic supplementary material, table S5.

2.4. Determination of gene copy numbers in transgenic mice

Transgene copy number was detected by a standard protocol as previously described [30]. Briefly, transgene copy number was estimated by real-time quantitative polymerase chain reaction (qPCR). The qPCR was performed using a SYBR Premix Ex Taq (Takara) and signals were detected with Applied Biosystems 7500 real-time PCR system (Thermo, Waltham, MA, USA). The conditions used in PCR reactions were as follows: initial denaturation at 95°C for 30 s; 40 cycles of 95°C for 5 s, 60°C for 34 s. The exogenous gene copies were calculated using the $2^{\Delta\Delta Ct}$ method. The primer information is presented in electronic supplementary material, table S5.

2.5. RT-PCR and qPCR analysis

Total RNA was isolated using TRIzol reagent (Thermo) and was immediately reverse-transcribed using a Prime Script RT reagent kit (Takara). The reverse transcription PCR (RT-PCR) was amplified using Ex Taq (Takara). The qPCR was performed using a SYBR Premix Ex Taq (Takara) and signals were detected with Applied Biosystems 7500 real-time PCR System (Thermo). Relative mRNA expression was calculated using the $2^{\Delta\Delta Ct}$ method. The primer information is presented in electronic supplementary material, table S5.

2.6. PUFA analysis

The PUFA analysis was performed as previously described [17]. Briefly, wild-type and transgenic mice were assessed through gas chromatography, as previously reported [31]. Fresh mouse tissues were homogenized through grinding in liquid nitrogen, and an aliquot of the tissue homogenate in a glass methylation tube was mixed with 2 ml of chloroform-methanol (2:1). The samples were dried using nitrogen at 60°C. The precipitate was dissolved in 2 ml of pure hexane for chromatographic assessment, and then methyl esterification was added in a 400 µl saturated KOH methanol solution, mixed well, vortexed for 5 min and centrifuged for 10 min at 2000 rpm min⁻¹. The gas chromatography coupled to mass spectrometry (GC-MS) analysis was performed using a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan), and the conditions were as follows: hp-88 column, helium gas carrier, a constant linear velocity of 20.0 cm s⁻¹, a segregation ratio of 20.0%, an injection volume of 1 µl, and a temperature programme of 60°C for 1 min, increased at 40°C min⁻¹, and held at 140°C for 10 min, increased at 4°C min⁻¹, and held at 240°C for 15 min to complete the run.

2.7. Statistical analyses

All experiments were repeated at least three times. One-way ANOVA was used to determine statistical significance following Dunnett’s test to determine the statistical significance between the relative group. Statistical analysis was conducted using Statistical Analysis System software (SAS Institute, Cary, NC, USA). All data were expressed as mean ± s.d. Differences were considered to be significant when $p < 0.05$.

3. Results

3.1. Generation of Fad3 transgenic mice

We microinjected 75 fertilized zygotes with linearized Fad3 transgenic expression cassettes (figure 1a). Two recipients transferred with a total of 60 embryos became pregnant and gave birth to 16 offspring (26.7%). The PCR genotyping analyses showed that 3 of the 16 mice were positive (18.8%; 1 male and 2 females; figure 1b,c). As estimating the transgene copy number is a significant step in transgenic animal research, we next investigated the Fad3 gene in the transgenic mice. The real-time qPCR, based on serial dilution curves, was used to determine the copy number of exogenous Fad3 in transgenic mice, and the glyceraldehyde-3-phosphate dehydrogenase gene (Gapdh) in mice was used as an endogenous reference gene. With a serial of dilutions, the standard curves of the threshold cycle (Ct) relative to the log of each initial template copy of Fad3 and Gapdh gene were obtained, and the correlation coefficients were 0.9996 and 0.9997, respectively (electronic supplementary material, figure S1b and table S1). The transgenic copy number was obtained by comparing the initial template copy of Fad3 with that of Gapdh. Among the three putative transgenic mice, one male had 13 copy numbers, and two females had...
14 and 5 copies, respectively (electronic supplementary material, table S2). In order to maintain the stability of the exogenous gene, the transgenic mice with the highest copies of Fad3 were selected as F0 generation. At 6 weeks old, the founder F0 mice were crossed to the littermates to produce F1 mice (three males and five females). To assess the potential expression of the Fad3 gene in vivo, we extracted the total RNA from F0 and F1 transgenic mice and analysed the mRNA by RT-PCR. As expected, the Fad3 mRNA could be detected in the skeletal muscle, fat, heart, liver, spleen, lung and kidney of the F0 and F1 transgenic mice (figure 1d).

### 3.2. Fad3 can convert n-6 PUFAs to n-3 PUFAs

To determine whether the plant-derived Fad3 gene can convert n-6 to n-3 PUFAs in transgenic mice, the major tissues from transgenic mice and wild-type mice were assessed for n-6 PUFA (LA, γ-LA and AA) and n-3 PUFA (ALA, EPA and DHA) levels. Gas chromatographic analysis showed that F0 Fad3 transgenic mice contained higher amounts of n-3 PUFAs and lower amounts of n-6 PUFAs compared with the wild-type mice (figure 2a,b; electronic supplementary material, table S3). In addition, the major tissues (skeletal muscle, fat, heart, liver, spleen, lung and kidney) from F1 transgenic mice were also collected and analysed for n-3 and n-6 PUFAs. There was at least 2.84-fold reduction of the n-6/n-3 ratio in F1 transgenic mice compared with wild-type mice (skeletal muscle: from 2.62 to 0.55, p < 0.05; fat: from 2.41 to 0.55, p < 0.05; heart: from 2.77 to 0.43, p < 0.05; liver: from 1.93 to 0.68, p < 0.05; spleen: from 2.76 to 0.62, p < 0.05; lung: from 2.31 to 0.42, p < 0.05 and kidney: from 2.13 to 0.65, p < 0.05; figure 2c; electronic supplementary material, table S3). Moreover, the ratio of n-6/n-3 PUFAs in the F1 transgenic mice was similar to the ratio in F0 (skeletal muscle: from 2.62 to 0.46, p < 0.05; fat: from 2.41 to 0.57, p < 0.05; heart: from 2.77 to 0.61, p < 0.05; liver: from 1.93 to 0.55, p < 0.05; spleen: from 2.76 to 0.98, p < 0.05; lung: from 2.31 to 0.62, p < 0.05 and kidney: from 2.13 to 0.47, p < 0.05; figure 2c; electronic supplementary material, table S3). These results indicate that the plant Fad3 gene can be functionally expressed in the major tissues of F0 and F1 transgenic mice, and Fad3 plays an active role in the conversion of n-6 into n-3 PUFAs.

### 3.3. Generation of Fad2–Fad3 double transgenic mice

Because Fad3 single transgenic mice lack the Fad2 enzyme and cannot de novo synthesize n-6 PUFAs, they must take it from plants or seafood in their diets (i.e. the synthesis of

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**Figure 2.** PUFA composition of Fad3 single transgenic mice. (a) Partial gas chromatogram traces showed fatty acid profiles of total lipid extracted from muscle tissues of wild-type and Fad3 single transgenic mice. (b) Comparison of the n-6 and n-3 PUFAs concentration between wild-type and Fad3 single transgenic mice in skeletal muscle, fat, heart, liver, spleen, lung and kidney. (c) Ratio of n-6/n-3 PUFAs in wild-type and Fad3 single transgenic mice. All the bars represent mean ± s.d., and different superscript letters (a–b) in each column represent statistically significant differences (p < 0.05), WT: wild-type.
n-3 PUFAs is limited by n-6 PUFAs). We therefore microinjected a total number of 78 fertilized embryos, collected from superovulated female mice, with linearized pCMV–Fad2–Fad3 expression cassettes (figure 3a). Two recipients receiving a total number of 60 embryos became pregnant and gave birth to 14 (14/60, 23.3%) offspring. Genotyping analysis of the genomic DNA from each offspring indicated that five (5/14, 35.7%) of the puppy mice were Fad2–Fad3 transgenic single transgenic (1 male and 4 females; figure 3b,c). Consistent with the strategy of single Fad3 single transgenic mice breeding, the Fad2–Fad3 mice were crossed with the positive littermate mice to produce F1 mice. To assess the expression potential of the plant Fad2–Fad3 transgene in vivo, we extracted the total RNA from major tissues, including skeletal muscle, fat, heart, liver, spleen, lung and kidney and analysed the mRNA by RT-PCR with specific primers for Fad2–Fad3 and housekeeping gene Gapdh. RT-PCR analysis indicated that the plant Fad2–Fad3 genes were expressed in F0 and F1 transgenic mice, while no PCR signal was detected in the non-transgenic littermates (figure 3d).

### 3.4. Fad2–Fad3 double transgenic mice can simultaneously produce both n-6 and n-3 PUFAs

To determine whether or not the double transgenic mice expressing the Fad2 enzyme catalysed the synthesis of n-6 PUFAs, the F0 double transgenic mice and non-transgenic littermates were used for PUFA analysis. The concentrations of total n-6 PUFAs (LA, γ-LA and AA) in major tissues of the Fad2–Fad3 mice were at least 1.27-fold higher than in the wild-type mice (figure 4a,b). Among them, LA, γ-LA and AA showed at least 1.24-fold, 1.71-fold and 1.43-fold increase in major tissues, respectively (figure 4c; electronic supplementary material, table S4). On the other hand, the concentration of total n-3 PUFAs (ALA, EPA and DHA) in the Fad2–Fad3 transgenic was at least 2.74-fold (skeletal muscle: from (5.18 ± 0.41) to (20.77 ± 1.38), p < 0.05; fat: from (7.48 ± 0.41) to (20.47 ± 1.37), p < 0.05; heart: from (3.67 ± 0.62) to (16.63 ± 0.24), p < 0.05; liver: from (6.05 ± 0.21) to (18.64 ± 0.89), p < 0.05; spleen: from (2.72 ± 0.76) to (9.54 ± 0.90), p < 0.05; lung: from (3.87 ± 0.16) to (14.09 ± 1.16), p < 0.05 and kidney: from (4.28 ± 0.75) to (15.55 ± 1.30), p < 0.05; figure 4d; electronic supplementary material, table S4). For the detailed analysis of n-3 PUFAs, the concentrations of ALA, EPA and DHA were significantly increased in Fad2–Fad3 mouse skeletal muscle (ALA: 5.76-fold; EPA: 5.05-fold; DHA: 3.83-fold), fat (ALA: 1.91-fold; EPA: 4.5-fold; DHA: 2.74-fold), heart (ALA: 1.72-fold; EPA: 4.15-fold; DHA: 4.83-fold), liver (ALA: 4.87-fold; EPA: 5.2-fold; DHA: 2.89-fold), spleen (ALA: 2-fold; EPA: 2.05-fold; DHA: 3.71-fold), lung (ALA: 2.91-fold; EPA: 5.22-fold; DHA: 3.64-fold) and kidney (ALA: 3.54-fold; EPA: 2.74-fold; DHA: 3.71-fold) compared with the wild-type mouse (figure 4d; electronic supplementary material, table S4).

To assess the function of the Fad2–Fad3 enzymes in the F0 and F1 transgenic mouse, the PUFA content in the major tissues was compared, including skeletal muscle, heart, liver, spleen, lung, kidney and fat. Figure 4b and electronic supplementary material, table S4 display the PUFA profile of seven tissues from F0 and F1 of the Fad2–Fad3 mouse. It shows that all the n-6 and n-3 PUFAs were higher when compared with wild-type mice, indicating that the Fad2–Fad3 double transgenic mice had efficiently converted the mono-unsaturated fatty acids into n-6 and n-3 PUFAs in their bodies.
muscle tissues of wild-type (WT) and Fad2–Fad3 double transgenic mice, as detected by qPCR (figure 5).

To verify the biological functions of Fad2 and Fad3 in the Fad2–Fad3 transgenic mice, we examined the expression level of fatty acid biosynthesis-related genes, including fatty acid synthase genes, fatty acid-binding protein and fatty acid oxidation genes. Compared with Fad3 single transgenic and the wild-type mice, the fatty acid synthesis genes including Fasn, Scd1 and Acc were significantly decreased in the Fad2–Fad3 double transgenic mice, as detected by qPCR (figure 5a). We also compared the fatty acid-binding protein (Fabp4) and fatty acid oxidation-related genes (Lipe, Lpl, Ppar-γ, Lcad) expression between different transgenic mice via qPCR. The qPCR results showed that the expression of fatty acid-binding protein and fatty acid oxidation-related genes were increased in Fad3 single and Fad2–Fad3 double transgenic mice compared with the wild-type mice (figure 5a).

To further consolidate the qPCR results, we compared the concentrations of total n-6 and n-3 PUFAs between different transgenic mice. The seven major tissues from Fad2–Fad3 double transgenic mice were collected and analysed for n-6:n-3 PUFA ratio. Notably, F0 and F1 double transgenic mice showed a substantially lower n-6:n-3 ratio in all tissues examined compared with wild-type mice (skeletal muscle: WT: 9:1, F0: 3.3:1, F1: 4.8:1).

### 3.5. Fad2–Fad3 double transgenic mice established their own PUFA biosynthetic pathways

To further consolidate the qPCR results, we compared the concentrations of total n-6 and n-3 PUFAs between different transgenic mice. The seven major tissues from Fad2–Fad3 double transgenic mice were collected and analysed for n-6:n-3 PUFA ratio. Notably, F0 and F1 double transgenic mice showed a substantially lower n-6:n-3 ratio in all tissues examined compared with wild-type mice (skeletal muscle: WT: 9:1, F0: 3.3:1, F1: 4.8:1).
4. Discussion

Except for C. elegans, animals (including humans) have not been reported to possess desaturase genes, which can change the ratio of n-6/n-3 fatty acids [1,4]. Mammals must obtain n-6 PUFAs and n-3 PUFAs from their daily diet. In order to enable humans to take in more health beneficial PUFAs from the dietary food, an increasing number of researchers began to produce transgenic livestock that carries fatty acid desaturase genes.

In recent years, we have produced Fat1 transgenic cows and sheep [18,22,23]. An analysis of fatty acids demonstrated that the Fat1 transgenic animals produced high levels of n-3 PUFAs and a significantly reduced n-6/n-3 PUFA ratio in their tissues and milk [21,23,32–34]. Lai et al. also reported that EPA (20 : 5, n-3) and DHA (22 : 6, n-3) levels in Fat1 transgenic pigs increased by 15-fold and 4-fold, respectively, and the n-6/n-3 PUFA ratio decreased by 80.2% [20,35]. Therefore, the n-3 PUFAs desaturase Fat1 can be functionally expressed in animals, or at least in cows, sheep and pigs. The n-3 PUFAs contents in Fat1 transgenic animals were significantly improved, which may make the n-3 PUFAs rich animal source food for human nutrition. However, transgenic animals prepared using C. elegans-derived Fat1 genes may create problems in terms of food safety [13].

Previous studies of Fat1 transgenic animals have mainly focused on accelerating PUFA transition from n-6 to n-3 PUFAs. Although Fat1 transgenic animals are rich in n-3 PUFAs, these animals lack n-6 PUFAs [21,23,32–34]. Deficiencies in n-6 PUFAs may cause reproductive failure, skin lesions, fatty liver, reduced growth and polydipsia [25]. To solve these problems, we used plant Fad2 gene, which can promote de novo biosynthesis of n-6 PUFAs [17,27]. Although the Fat1 gene has been widely used in transgenic animals, related research and application of the Fat1 gene have not been widely reported.

In the current study, we successfully produced transgenic mice that carried single Fat3 or double Fad2–Fat3 genes. Our results indicate that the plant-derived Fad transgenic system can endogenously synthesize PUFAs in a transgenic animal. In the Fat3 single transgenic mice, we found that
the concentration of the n-6 PUFAs was decreased and that of the n-3 PUFAs was increased compared with wild-type mice. By contrast, in Fad2–Fad3 double transgenic mice, the concentration of both n-6 and n-3 PUFAs was significantly increased compared with Fad3 single transgenic and wild-type mice. Thus, the Fad2–Fad3 double transgenic mice have their own PUFAs biosynthetic pathways. These Fad transgenic mice provide an animal model with which to study the mechanism of fatty acid biosynthesis. In addition, the generation of Fad2–Fad3 double transgenic livestock that produces PUFAs may be an economical and safe way to produce PUFAs-rich food.

Ethics. All animal experiments were approved by the Animal Care and Use Committee of Inner Mongolia University and were performed in accordance with the Animal Research Institute Committee guidelines.

Data accessibility. This article has no additional data.

Authors’ contributions. G.L. conceived of and designed the study. L.S., L.Y., J.W., X.L., L.B. and A.D. performed the experiments and analysed the data. G.L. and L.Y. supervised the project. L.S., L.Y. and G.L. wrote the manuscript. All authors read and approved the final manuscript.

Competing interests. All authors declare no competing interests.

Funding. This study was supported by the Genetically Modified Organisms Breeding Major Projects (grant no. 2016ZX08007-002), the opening project of State Key Laboratory of R2BGL (to L.Y.), the Inner Mongolia Autonomous Region Basic Research Project (to G.L.) and Inner Mongolia Science and Technology Innovation Guide Project (KCBJ2018002).

Acknowledgements. We are grateful to our colleagues in the laboratory for their assistance with the experiments and in the preparation of this manuscript.

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