

Transgenic Pigs Carrying a Synthesized Fatty Acid Desaturase Gene Yield High Level of ω -3 PUFAs

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Abstract

Polyunsaturated fatty acids (PUFAs) are essential for normal growth in mammals, especially the ω -3 PUFAs, which play important roles in preventing several life-threatening diseases, such as coronary heart disease and diabetes. In this study, we aimed to investigate whether the *sFat-1* gene from *Caenorhabditis briggsae* could be functionally expressed in transgenic pigs, and whether the transgenic could synthesize high quality ω -3 PUFAs endogenously. In this study, a gene construct consisting of CMV promoter and 1.9 kb cDNA of ω -3 fatty acid desaturase gene (*sFat-1*) from *C. briggsae* was injected into the male pronucleus of pig embryos by microinjection. The piglets were screened for the transgene by PCR, Southern blot and reverse transcription-PCR analysis. Pigs that give positive results were mated with wild-type pigs to produce the next generation and the transmission of transgene was examined by PCR analysis. Fatty acids compositions of various tissues in the transgenic pigs were then analyzed by gas chromatograph. A total of 878 embryos were transferred into 42 recipients, among which 29 successfully got pregnant and gave birth to a total of 162 piglets, and 8 of them were identified to be transgenic. Fatty acid compositions in the transgenic pigs were altered, and the levels of ω -6: ω -3 ratios were decreased from 14.53 in the control to 2.62 in *Fat-1* transgenic pigs. A number of primary *sFat-1*-transgenic pigs were bred in this study, which lays the foundation for cultivation of new varieties of transgenic pigs.

Key words: transgenic pigs, *sFat-1* gene, ω -3 polyunsaturated fatty acids, microinjection

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are essential fatty acids for mammals but cannot be synthesized *in vivo* (Clandinin *et al.* 1985; McLennan *et al.* 1988). PUFAs play important roles in regulating lipid metabolism, preventing and treating cardiovascular and cerebrovascular diseases, and resisting cancer (Goodnight *et al.* 1981). Based on the location of the double bond in the

carbon chain, PUFAs can be assigned into two types: ω -6 fatty acids and ω -3 fatty acids. The ω -3 PUFAs are remarkably effective for promoting cerebral and retinal development (Neuringer *et al.* 1986), preventing and treating cardiovascular and cerebrovascular diseases (Hu *et al.* 2003), and inhibiting tumor cell proliferation (Xia *et al.* 2005), while the ω -6 PUFAs will contrarily lead to platelet aggregation and thrombosis and promote tumor cell proliferation in animals (Das 2008). Furthermore, the balance of the ω -6/ ω -3 fatty

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acid proportions is of utmost importance in the maintenance of homeostasis and normal development in individuals (Borsonelo and Galduroz 2008). Unfortunately, along with the development of industrialized and intensive farming, the content of ω -6 PUFAs gradually increases in livestock and poultry products, while the content of ω -3 fatty acids decreases in artificially cultivated vegetables, which results in an imbalance of proportions between the intake of ω -6 and ω -3 fatty acids in the daily diet of humans. It is generally believed that the optimal ratio of *in vivo* ω -6: ω -3 should be between 4:1 and 6:1, but in fact, the ratio approaches 18:1 in Western diets, leading to a comparatively high incidence of cardiovascular diseases (Simopoulos 2002). Therefore, measures developed to balance the ratio of ω -3 PUFA intake in diets is of great importance to human health.

The ω -3 fatty acid desaturase gene is a key gene for ω -3 PUFA synthesis, which could catalytically convert 18-20 carbons ω -6 PUFAs to ω -3 PUFA by adding an unsaturated bond on the third carbon of its carbon chain (Pereira *et al.* 2003; Leonard *et al.* 2004). In the past twenty years, this gene had been widely used in transgenic mammal production (Spychalla *et al.* 1997; Kang *et al.* 2001; Ge *et al.* 2002; Kang *et al.* 2004; Lai *et al.* 2006; Li *et al.* 2006). In 2004, Saeki *et al.* (2004) transferred a Δ 12 fatty acid desaturase gene from spinach into pigs, successfully resulting in the *in vivo* expression of a plant gene in mammals for the first time; meanwhile, they found that the content of linoleic acids (ω -6) in preadipocytes differentiated from transgenic pigs was 10-time higher than that in the control group. After isolating the *fat-1* gene from *Caenorhabditis elegans* and functionally expressed the ω -3 fatty acid desaturase gene in mammalian cells through adenovirus mediation (Kang *et al.* 2001), Kang *et al.* (2004) found that the intracellular content of ω -3 PUFAs in transgenic mice expressing *Fat-1* gene increased and the ω -6: ω -3 ratio reduced from 15:1 to 1:1. In 2006, Lai *et al.* (2006) successfully generated cloned transgenic pigs rich in ω -3 PUFA, they found that the cloned pigs had higher *in vivo* content of ω -3 PUFAs compared with the control group.

In 2006, Zhu *et al.* (2008) synthesize the cDNA se-

quence of *sFat-1* gene from revised and optimized codon based on *Caenorhabditis briggsae*, their research results showed that the synthesized *sFat-1* gene possess functional activity and greater capability of producing ω -3 PUFAs in transgenic mice. The most important results of their research is that the longer carbon chain ω -3 PUFAs, such as DHA (22:6 n-3) and DPA (22:5 n-3), were greatly increased in transgenic mice. Their studies give us great inspiration. The objective of this study was to investigate whether the *Fat-1* gene from *C. briggsae* could be functionally expressed in transgenic pigs, and whether the transgenic pigs could produce high-quality ω -3 PUFAs, especially DHA and DPA.

The results of this research showed that *sFat-1* was highly expressed in transgenic pigs and remarkable changes were detected in the ω -6: ω -3 ratio of transgenic pigs from 21.05 to 11.86%. This is the first reported case of *sFat-1*-transgenic pigs produced in China, laying the foundation for further investigation of the genetic stability of foreign genes in transgenic pigs and the safety of transgenic animals.

MATERIALS AND METHODS

Construction of the *sFat-1* expression vector

The *sFat-1* cDNA sequence of optimized codons from *C. briggsae* was cloned into the pCAGGS expression vector (Fig. 1) containing the CAG promoter, which consists of the CMV enhancer, chicken β -actin promoter that can drive high expression of the gene in a variety of animal tissues. After extraction and purification with a Qiagen Plasmid Endofree Maxi Kit (Qiagen, Valencia, CA, USA), the constructed expression vector was linearized by digestion with *Bam*H I and *Sal* I, and the 3 685 bp target fragments that contained the CAG promoter, *sFat-1* coding sequence and rabbit β -globulin polyA termination sequence were recovered and dissolved in TE-buffer (10 mmol L⁻¹ Tris-HCl, 1 mmol L⁻¹ EDTA, pH 7.4). Once the final concentration of the solution reached 3 μ g mL⁻¹, the sample was centrifuged at 13 000 \times g for 30 min at 4°C prior to microinjection following filtration with a 0.22 μ m filter and a small amount of supernatant liquid was taken for microinjection.

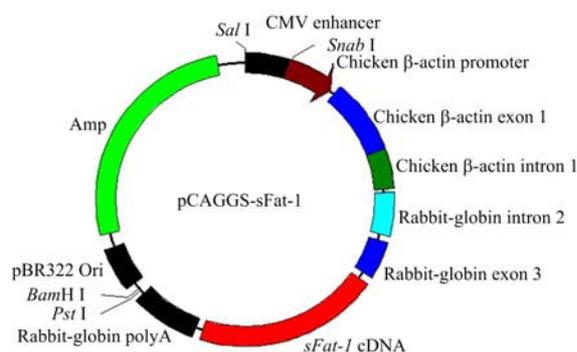


Fig. 1 Construction of the pCAGGS-sFat-1 expression vector.

Production of transgenic pigs

Transgenic pigs were produced as described previous (Hammer *et al.* 1985).

Fertilized eggs were collected from Hubei White pigs at 5 mon of age. At the d 15-18 of each estrus cycle, gilts were superovulated with PMSG (18 IU kg⁻¹) and HCG (800 IU per pig) through intravenous or intramuscular injection, and zygotes in 1-cell stage were collected through a small opening on the pig abdomen by flushing embryos out of the oviduct using Dulbecco's phosphate-buffered saline (D-PBS). The flushed out embryos were centrifuged at 15 000×g for 3 min and well-developed cell embryos were selected for microinjection (Wall *et al.* 1985). DNA was injected into the male pronucleus of fertilized eggs. Injected embryos were transferred into synchronized estrus recipient sows. The breeder sows were reared under normal conditions prior to delivery.

Identification of transgenic pigs

PCR analysis A tail biopsy from each piglet at birth was performed and the genomic DNA was extracted from the tail tissues for gene integrity analysis using PCR and Southern blotting (Matsumoto *et al.* 1993). The PCR was performed with primers *sFat-1*-PL (5'-GTA TGG TCG CTC ATT CCT-3') and *sFat-1*-PR (5'-GCT TCC CAA TCC TTA TCC TG-3') in a 20 μ L reaction volume consisting of 1× buffer (TaKaRa, Japan), 50 ng DNA template, 75 μ mol L⁻¹ each dNTP, 0.3 μ mol L⁻¹ each primer, 1.5 mmol L⁻¹ MgCl₂ and 1.0 U *Taq* DNA polymerase (TaKaRa, Japan). The thermal cy-

cling parameters for the PCR were 95°C for 5 min, then 35 cycles at 95°C for 30 s, 54°C for 30 s and 72°C for 30 s, and then a final extension step at 72°C for 10 min. The PCR products were analyzed on a 1.5% (w/v) agarose gel stained with GelGreen (Biotium, USA). **Southern blot analysis** Genomic DNA (10 μ g) from PCR-positive pigs was digested with *Hind* III and *Xho* I enzymes, and separated by 0.8% (w/v) agarose gel electrophoresis at low voltage overnight at 4°C, and then the separated DNA was transferred to positively charged nylon membranes and fixed by UV cross-linking for 30 s. Southern blotting was carried out according to the manufacturer's instruction for the DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany) with a DIG-labeled 556 bp fragment amplified using primers *sFat-1*-PL and *sFat-1*-PR.

Analysis of the founders with RT-PCR and production of the next generation Tail tissues of founders were collected and total RNA extracted with Trizol (Invitrogen Inc., USA) in accordance with the manufacturer's instructions. The mRNAs were then reverse-transcribed with M-MLV using oligo-dT primers in a 50 μ L reaction. The PCR was performed in a 20 μ L volume with primers *sFat-1*-PL and *sFat-1*-PR; the expected PCR product was about 556 bp, and the pig *GAPDH* gene was used as an internal control. Pigs that gave positive results were mated with wild-type pigs to produce the next generation, and transmission of the transgene to piglets was determined by PCR analysis of genomic DNA extracted from tail tissues as described above.

Lipid analysis Lipids were extracted from tissues of 8 transgenic pigs with chloroform and methanol as described (Sukhija and Palmquist 1998), and the fatty acid compositions were analyzed with gas chromatography-mass spectrometry (GC-MS). Tissues of transgenic and control pigs were cut into small pieces and freeze dried, 50-500 mg dried tissues (contains 10-50 mg fatty acids) were accurately weighted and transferred into a 50-mL test tube. Add 5 mL internal standard solution (1 mg mL⁻¹ methyl nonadecanoate) and 4 mL acetyl chloride/methanol into the tube, vortex the mixture for 1 min and centrifuged it at 2 500 r/min for 5 min, the top phase were removed carefully into a new clean glass tube used for analysis. The fatty acid methylesters (FAME) were quantified using HP6800 gas chromato-

graph equipped with a HP-INNWAX chromatogram column (30 m×320 μm×0.5 μm). The GC-MS injector and detector were maintained at 250 and 280°C. The oven program increased from 50 to 220°C, maintained for 10 min, and finally rose to 250°C at the rate of 10°C min⁻¹, maintained for 5 min. Relative contents of fatty acids were calculated using the areas of various fatty acids.

Statistical analysis Statistical analysis was performed using SPSS software under *t*-test.

RESULTS

Generation of transgenic pigs and expression of the *sFat-1* gene from *C. briggsae*

Zygotes collected from superovulated gilts were micro-injected with DNA construct, and injected embryos were transferred into the oviducts of recipient animals, 70% (29/42) of the recipients became pregnant and gave birth to 162 piglets. PCR and Southern blotting were used to analyze the genomic DNA from each piglet; with the results indicating that 8 of the piglets were transgenic (Figs. 2 and 3). All the transgenic piglets grew up to be healthy. When the transgenic piglets were 2-mon-old, total RNA was extracted from tail tissues and the RNA analyzed by RT-PCR in 8 founder pigs. The RT-PCR results revealed *sFat-1* mRNA in all founders examined (Fig. 4). After puberty, the transgenic pigs with active transgene expressing were mated with wild-type pigs and produced 144 piglets; PCR analysis indicated that 69 of the piglets were transgenic (48%).

Elevated levels of ω-3 fatty acids in tissues of transgenic pigs

To examine the function of *sFat-1* in transgenic pigs, the fatty acid composition from their muscle tissues was determined by GS-MS analysis. As shown in Table, the levels of ω-3 PUFAs (18:3 ω-3, 20:5 ω-3, 22:5 ω-3, and 22:6 ω-3) were ≈3.1-fold higher in the muscle tissues of transgenic pigs compared with wild-type muscle tissues ($P<0.01$), and the ω-6:ω-3 ratios were decreased from 14.53 in the control to 2.62 in *sFat-1* transgenic pigs ($P<0.01$).

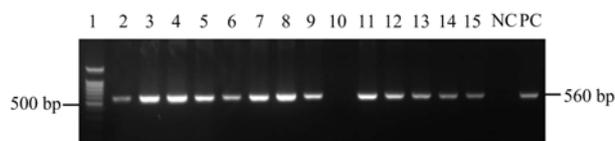


Fig. 2 Identification of transgenic pigs by PCR. Line 1, 100 bp DNA ladder; lines 2-15, transgenic founder pigs; NC, wild-type nontransgenic littermates pig; PC, positive control. The same as below.

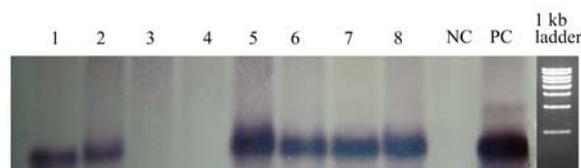


Fig. 3 Results of Southern blotting for transgenic pigs. Lines 1-8, transgenic founder pigs.

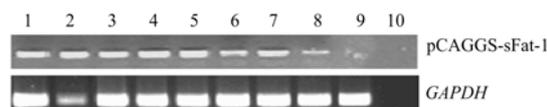


Fig. 4 Expression of the *sFat-1* gene was detected by RT-PCR in tail tissues of pCASSG-sFat-1 transgenic pigs. Total RNA was extracted from the tail tissues of transgenic founders and wild-type pigs. The *GAPDH* gene was used as an internal control. Lanes 1-8, transgenic founders; lane 9, negative control; lane 10, blank control.

Table Fatty acid composition of partial lipids from the longissimus dorsi muscle of *sFat-1* transgenic pigs and wild-type nontransgenic littermates

Fatty acids ¹⁾	Transgenic	Wild-type
18:2 n-6	11.01±1.80	18.85±1.03*
18:3 n-6	0.06±0.01	0.05±0.01
20:4 n-6	0.63±0.05	1.73±0.04*
22:4 n-6	0.16±0.01	0.42±0.06*
18:3 n-3	2.50±0.02	0.95±0.05*
20:5 n-3	0.98±0.10	0.06±0.02*
22:5 n-3	0.70±0.04	0.34±0.02*
22:6 n-3	0.41±0.04	0.11±0.02*
Sum of n-6 PUFAs	11.86±1.86	21.05±1.12*
Sum of n-3 PUFAs	4.60±0.18	1.46±0.09*
n-6/n-3	2.62±0.48	14.53±1.66*

¹⁾ The concentration of fatty acids is presented as the percentage of total fat in the longissimus dorsi muscle samples from 8 6-mon-old *sFat1* transgenic pigs and 8 control pigs. All results are given as the mean±SD.

*, significantly different ($P<0.01$) between transgenic and control pigs.

DISCUSSION

In this study, we established transgenic pigs express-

ing *sFat-1* gene by microinjection method. Microinjection method is the least complicated and most reliable method available for producing transgenic animals compared to other methods, such as sperm-mediated gene transfer method and somatic cell nuclear transplantation technology, while its limitation is also obvious, one of the hallmarks is the random integration of transgene (Wall 2001). If the transgene is integrated into one of the chromosome sites that are lethal to embryos, none or few transgenic products could be produced; if the transgene integrated into the coding regions of the genome that are important for the normal growth or development of animals, and then the transgenic offspring will become abnormal (Woychik *et al.* 1985). In our study, the transgene *sFat-1* could be detected from the genomic DNA of transgenic pigs, and the gene product indeed have a significant effect on changing the fatty acid compositions of pigs, these results demonstrated that the transgene integration site is safe for the pig, we called this “friendly locus”. This finding gives us great inspiration: the *sFat-1* transgene integration site of these transgenic pigs in our study might become a friendly-locus used for transgenic animal production in the future. In order to achieve this objective, detailed investigation about the integration site and gene copy number of *sFat-1* gene needed to be further studied.

ω -PUFAs have been verified to exert promising role in preventing and therapying diseases. However, mammals are incapable of synthesizing ω -3 PUFAs due to the lacking of a fatty acid desaturase gene, which could add a double bond in specific location of the ω -6 PUFAs carbon chains to convert it into ω -3 PUFAs. So the levels of ω -PUFAs in their body mainly depend on dietary intake. While PUFAs are easily oxidized and liable to release foreign odors, therefore they are not conducive to processing and storage and will affect the flavor and fatty acid composition of pork products. Therefore, many studies have been conducted in recent years to elevate the content of ω -3 PUFAs by biotechnological methods without increasing the total *in vivo* content of PUFAs in animals. In previous studies, researchers mainly focused on using *Fat-1* gene from *C. elegans* to produce transgenic animals, and their research results showed that the product of this gene could produce abundant ω -3 PUFAs with 18-22 carbons. Although, people are seeking other desaturase

genes that could be used for transgenic domestic animal producing for the purpose of increasing the fatty acid composition of food products. *sFat-1* gene was first found and cloned from the roundworm *C. briggsae*, and its CDS sequence was revised according to the “codon bias” rules (Gustafsson *et al.* 2004). Transgenic mice expressing *sFat-1* gene yield higher levels of ω -3 PUFAs this positive results prompted us to further explore the function of this gene in domestic animals. When we introduced this *sFat-1* gene into pigs, the same pleasant results was obtained, the levels of ω -3 PUFAs, especially the DHA (C22:6 n-3) and DPA (C22:5 n-3) in our transgenic pigs increased greatly.

This is the first report of producing transgenic pigs using the *sFat-1* gene from *C. briggsae*, and these transgenic pigs will used as animal models for further studying the role of ω -3 PUFAs in the prevention of many human disease.

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