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Generation of cloned transgenic pigs rich in omega-3 fatty acids

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Abstract

Meat products are generally low in omega-3 (*n*-3) fatty acids, which are beneficial to human health. We describe the generation of cloned pigs that express a humanized *Caenorhabditis elegans* gene, *fat-1*, encoding an *n*-3 fatty acid desaturase. The *hfat-1* transgenic pigs produce high levels of *n*-3 fatty acids from *n*-6 analogs, and their tissues have a significantly reduced ratio of *n*-6/*n*-3 fatty acids ($P < 0.001$).

The health benefits of long chain *n*-3 fatty acids, found mainly in fish oils, are well recognized. Meat products normally contain small amounts of *n*-3 fatty acids and large amounts of *n*-6 fatty acids¹. Diets with a high ratio of *n*-6/*n*-3 fatty acids may contribute to the prevalence of many diseases, such as coronary artery disease, cancer, diabetes, arthritis and depression². The high *n*-6/*n*-3 ratio in meat products is largely due to the extensive use of grains rich in *n*-6 fatty acids but deficient in *n*-3 fatty acids as animal feed. In addition, livestock cannot convert *n*-6 fatty acids into *n*-3 fatty acids because they lack an *n*-3 fatty acid desaturase gene, such as the *fat-1* gene found in the roundworm *C. elegans*³. Earlier work in transgenic mice carrying the *fat-1* gene has suggested the feasibility of creating *fat-1* transgenic livestock capable of producing *n*-3 fatty acids from the corresponding *n*-6 fatty acids⁴. Here we report the cloning of *fat-1* transgenic pigs that produce high levels of *n*-3 fatty acids in their tissues and organs.

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COMPETING INTERESTS STATEMENT

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An *hfat-1* expression vector, pCAGGS-*hfat-1*, which contains a humanized *fat-1* cDNA (with modification of codon usage) driven by the cytomegalovirus enhancer and chicken β -actin promoter, has been described previously⁴. A pgk-neo expression cassette as a selection marker was inserted into pCAGGS-*hfat-1* to generate pST103, which was transfected into early-passage male primary porcine fetal fibroblast cells, pCFF4-3⁵, by electroporation; the transfected cells were selected with 250 μ g/ml G418. The G418-resistant colonies were pooled. Gas chromatographic analysis showed that pCFF4-3/pST103 cells contained higher amounts of *n*-3 fatty acids and lower amounts of *n*-6 fatty acids compared with the nontransfected pCFF4-3 cells, indicating that the *hfat-1* protein was functional in the primary porcine cells. The pCFF4-3/pST103 cells were used to clone *hfat-1* transgenic pigs by nuclear transfer as described previously⁶. A total of 1,633 reconstructed embryos were transferred into 14 gilts that exhibited a natural estrus. Twelve early pregnancies were established, and five of them went to term. Twelve (ten alive and two dead) male piglets were born by either caesarean section or natural delivery. PCR analysis of DNA samples from the tails of ten live piglets showed that six piglets (nos. 1, 3–5, 8 and 9) were positive for the *hfat-1* transgene whereas the others (nos. 2, 7, 10 and 11) were negative. Most piglets appeared normal at birth, and there was no obvious difference in appearance between the transgenic and nontransgenic littermates (**Supplementary Fig. 1** online). Commercial standard diets were given to recipients (gestation diet) and piglets (milk replacer).

Among the six *hfat-1* transgenic piglets, piglets nos. 4, 5 and 8 showed substantial differences in the fatty acid profile of their umbilical cord, tail and ear samples compared with their nontransgenic littermates. Because the piglets were cloned from a pool of over 100 G418-resistant colonies containing varying copy numbers of *hfat-1* at various integration sites, there was a wide range of expression of *hfat-1* among the transgenic piglets. Figure 1 displays the fatty acid profile of tail samples from transgenic piglet no. 4 and nontransgenic piglet no. 2. It shows that all four *n*-3 fatty acid peaks were higher and all four *n*-6 peaks were lower in piglet no. 4 compared with piglet no. 2, indicating that the *hfat-1* transgenic piglets had efficiently converted *n*-6 fatty acids into *n*-3 fatty acids in their bodies.

Piglets nos. 2, 4 and 5 were killed at 3 weeks of age because they developed symptoms of heart failure caused primarily by an interatrial septal defect. This defect has been reported in other cloned pigs⁶ and appears to be a function of the cloning process (incomplete nuclear reprogramming) rather than the *hfat-1* transgene: transgenic piglets nos. 8 and 9 did not have cardiac defects whereas nontransgenic piglet no. 2 showed the same symptoms as transgenic piglets nos. 4 and 5. In addition, *hfat-1* transgenic mice produced by pronuclei microinjection⁴ have been bred for many generations and show no heart failure symptoms (Kang *et al.*, unpublished data). This speculation was further supported by characterization of a litter of eight healthy male piglets that were cloned from muscle fibroblasts isolated from piglet no. 4. As expected, all of them were *hfat-1* transgenic. However, none showed heart failure symptoms at 3 weeks of age. Table 1 shows the fatty acid profiles of tail samples from these eight transgenic piglets and eight age-matched, wild-type, control piglets. The concentrations of total *n*-3 fatty acids in tail tissues of the transgenic piglets were threefold higher than in the wild-type piglets. Among them, eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) showed a 15-fold and fourfold increase, respectively. On the other hand, the concentration of total *n*-6 fatty acids in the transgenic piglets was reduced by 23%. Consequently, there was a fivefold reduction of the *n*-6/*n*-3 ratio in *hfat-1* transgenic piglets compared with wild-type piglets (from 8.52 to 1.69, $P < 0.001$) (Table 1).

The major tissues (muscle, liver, kidney, heart, spleen, tongue, brain and skin) from *hfat-1* transgenic piglets nos. 4 and 5 and nontransgenic piglet no. 2 were collected and analyzed for *n*-3 and *n*-6 fatty acids. Piglets nos. 4 and 5 showed a substantially lower *n*-6/*n*-3 ratio in all tissues examined compared with piglet no. 2 (**Supplementary Table 1** online). It was also

shown that piglet no. 4 had a lower *n*-6/*n*-3 ratio than piglet no. 5 in most of the tissues examined, indicating the heterogeneity of the cells used for nuclear transfer. The total *n*-3 fatty acids (alpha-linolenic acid (ALA), EPA, DPA and docosahexaenoic acid (DHA)) in skeletal muscle from the transgenic pigs were about 8% of total muscle fat on average, which is much higher than those in wild-type pigs (1–2%). Thus, these results indicate that *hfat-1* transgenic pigs can produce meat enriched in *n*-3 fatty acids.

Normal pork fat contains ~40% oleic acid, ~15% *n*-6 fatty acids and very little *n*-3 fatty acids (~1%). Several groups have reported the production of *n*-3 fatty acid-enriched pork by feeding pigs with flaxseed, fish oil or fish meal⁷. Total *n*-3 fatty acids increased from 1% to 6% of muscle fat, a yield of 250 mg per 100 g of fresh meat. These results showed that an increase in ALA (18:3 *n*-3) alone in pork can affect its sensory qualities. However, an increase in long chain *n*-3 fatty acids (EPA and DHA) did not affect the quality of freshly cooked pork.⁷ Pork quality can be affected by high intake of polyunsaturated fatty acids (PUFA) in the diet because it decreases the oleic acid content, increases the iodine value (hence oxidizability) and causes undesirable softening and yellowing of the carcass fat⁷. This is unlikely to be an issue in the *hfat-1* transgenic pigs because they only convert *n*-6 PUFA to *n*-3 PUFA and do not need extra PUFA in their diet.

The demand for *n*-3 fatty acids has increased considerably in recent years as evidence of their beneficial effects has grown. So far, the only way to enrich the tissues of mammals with *n*-3 fatty acids has been dietary provision of *n*-3 fatty acids. Thus, the food industry must feed animals with flaxseed, fish meal or other marine products. In view of the decline in marine fish stocks and the potential contamination of fish products with mercury and other chemicals, alternative, land-based dietary sources of *n*-3 fatty acids are needed. Generation of *fat-1* transgenic livestock that produce *n*-3 fatty acids may be an economical and sustainable strategy to address this need. In addition, *fat-1* transgenic pigs provide a large animal model in which to study the role of *n*-3 fatty acids in the prevention and treatment of various clinical conditions, such as coronary heart disease and immune-mediated disorders.

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Figure 1.

Partial gas chromatograph traces showing the polyunsaturated fatty acid profiles of total lipids extracted from tail tissues of a wild-type piglet (WT, piglet no. 2) and a *fat-1* transgenic piglet (Fat-1, piglet no. 4). Both animals were 1 week old and fed with the same milk replacer. Note the levels of *n*-6 polyunsaturated acids (18:2*n*-6, 20:4*n*-6, 22:4*n*-6 and 22:5*n*-6) are lower whereas *n*-3 fatty acids (marked with *, 18:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3) are abundant in the transgenic piglet no. 4 as compared with the nontransgenic piglet no. 2, in which there is very little *n*-3 fatty acid.

Table 1

n-3 and *n*-6 fatty acids concentration and *n*-6/*n*-3 ratios in tail samples from *hfat-1* transgenic and wild-type piglets

Fatty acids in tails ^a	Transgenic piglets (<i>n</i> = 8)	Wild-type piglets (<i>n</i> = 8)
ALA (18:3 <i>n</i> -3, %)	0.94 ± 0.10	0.63 ± 0.04
EPA (20:5 <i>n</i> -3, %)	4.21 ± 0.60	0.26 ± 0.07
DPA (22:5 <i>n</i> -3, %)	1.69 ± 0.19	0.35 ± 0.05
DHA (22:6 <i>n</i> -3, %)	1.75 ± 0.23	0.95 ± 0.21
Total <i>n</i> -3 FA (%)	8.59 ± 0.84	2.18 ± 0.25
Total <i>n</i> -6 FA (%)	14.28 ± 1.31	18.46 ± 1.41
<i>n</i> -6/ <i>n</i> -3 ratio	1.69 ± 0.30	8.52 ± 0.62

Fatty acids concentration is presented as percentage of total fat in the tail samples from eight 2-d-old *hfat-1* transgenic piglets and eight age-matched wild-type piglets. Total *n*-6 was calculated from linoleic acid (LA, 18:2 *n*-6) and arachidonic acid (AA, 20:4 *n*-6).

Total *n*-3 fatty acids was calculated from α -linolenic acid (ALA, 18:3 *n*-3), eicosapentaenoic acid (EPA, 20:5 *n*-3), docosapentaenoic acid (DPA, 22:5 *n*-3), and docosahexaenoic acid (DHA, 22:6 *n*-3). Each value represented the mean ± standard deviation from eight tail samples in each group with two independent measurements for each sample.

^aStatistical analysis with two tailed *t*-test shows there are Significant differences between the transgenic and the wild-type piglet samples ($P < 0.001$).