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Research paper

Transgenesis of humanized *fat1* promotes n−3 polyunsaturated fatty acid synthesis and expression of genes involved in lipid metabolism in goat cells

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ABSTRACT

The n−3 fatty acid desaturase gene *fat1* codes for the n−3 desaturase enzyme, which can convert n−6 polyunsaturated fatty acids (PUFAs) to n−3 PUFAs. The n−3 PUFAs are essential components required for normal cellular function and have preventive and therapeutic effects on many diseases. Goat is an important domestic animal for human consumption of meat and milk. To elevate the concentrations of n−3 PUFAs and examine the regulatory mechanism of *fat1* in PUFA metabolism in goat cells, we successfully constructed a humanized *fat1* expression vector and confirmed the efficient expression of *fat1* in goat ear skin-derived fibroblast cells (GEFCs) by qRT-PCR and Western blot analysis. Fatty acid analysis showed that *fat1* overexpression significantly increased the levels of total n−3 PUFAs and decreased the levels of total n−6 PUFAs in GEFCs. In addition, qRT-PCR results indicate that the *FADS1* and *FADS2* desaturase genes, *ELOV2* and *ELOV5* elongase genes, *ACO* and *CPT1* oxidation genes, and *PPARa* and *PPARγ* transcription factors are up-regulated, and transcription factors of *SREBP-1c* gene are down-regulated in the *fat1* transgenic goat cells. Overall, *fat1*-overexpression resulted in an increase in the n−3 fatty acids and altered expression of PUFA synthesis related genes in GEFCs. This work lays a foundation for both the production of *fat1* transgenic goats and further study of the mechanism of *fat1* function in the PUFAs metabolism.

1. Introduction

The n−3 polyunsaturated fatty acids (PUFAs), primarily eicosapentaenoic acid (20:5n−3, EPA), docosapentaenoic acid (22:5n−3, DPA) and docosahexaenoic acid (22:6n−3, DHA), are essential components required for the normal cellular function and play important roles throughout life—from early pregnancies to adulthood(Pang et al., 2014). They have preventive and therapeutic effects on a wide range of diseases, such as type-2 diabetes, coronary artery disease and hypertension, neurological or autoimmune disorders to cancer(Kris-Etherton et al., 2003; Whelan and Rust, 2006; Algamas-Dimantov et al., 2014; Kemse et al., 2014). While there is increasing evidence that excessive intake of n−6 PUFAs, mainly linoleic acid (18:2n−6, LA) and arachidonic acid (20:4n−6, ARA), leads to cancer, cardiovascular diseases and various mental disorders(Riediger et al., 2009; Lopez-Huertas, 2010; Wu et al., 2012), an optimal n−6/n−3 ratio in body lipids is essential for the normal growth and development, and plays an important role in the prevention and treatment of many clinical problems(Kang et al., 2001).

The n−3 PUFAs are synthesized through an alternating series of desaturations and elongations. Unfortunately, all mammals, including human beings and livestock, have lost the capacity to synthesize n−3 PUFA de novo (Nakamura and Nara, 2004). Furthermore, the n−3 and n−6 PUFAs are not interconvertible in mammalian cells (Goodnight et al., 1981); thus n−3 PUFAs are considered essential fatty acids in the human diet. Several studies have shown that the *Caenorhabditis elegans fat1* gene encoding an n−3 fatty acid desaturase, is responsible for the conversion of n−6 PUFAs to the corresponding n−3 PUFAs (Kang et al., 2001; An et al., 2012; Heerwagen et al., 2013; Pang et al., 2014). However, there are few studies designed to

Abbreviations: ACO, acyl-coenzyme A oxidase; ARA, arachidonic acid; CPT1, carnitine palmitoyltransferase 1; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; ELOVL2, elongase-2; ELOVL5, elongase-5; EPA, eicosapentaenoic acid; FADS1, fatty acid desaturase-1; FADS2, fatty acid desaturase-2; GEFCs, goat ear skin-derived fibroblast cells; LA, linoleic acid; PPARα, peroxisome proliferator-activated receptor alpha; PPARγ, peroxisome proliferator-activated receptor gamma; PUFAs, polyunsaturated fatty acids; SREBPF-1c, sterol regulatory element-binding protein-1c.

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investigate whether this *fat1* gene can be expressed functionally in goat cells (Kang, 2005). Moreover, almost all the existing research work concentrates on testing the effect of increasing exogenous PUFA levels in diets or cells on the expression of genes involved in PUFA metabolism, such as the fatty acid desaturase-1(*FADS1*) and desaturase-2 (*FADS2*), the elongases-2 (*ELOV2*) and elongases-5 (*ELOV5*), and the transcription factor sterol regulatory element-binding protein-1 (*SREBP-1c*) and peroxisome proliferator-activated receptor alpha (PPARs)(Tu et al., 2010; Ebrahimi et al., 2014), and fatty acid oxidation gene of acyl-coenzyme A oxidase (*ACO*) and carnitine palmitoyltransferase 1(*CPT1*), while the molecular mechanisms underlying the effect of overexpression *fat1* on PUFA endogenous synthesis have not been well-elucidated.

In this study, we successfully constructed a humanized *fat1* expression vector and examined whether the fat1 protein can be expressed in goat ear fibroblast cells (GEFCs) and whether the expression of the enzyme can exert a significant effect on cellular PUFAs. Additionally, we have also analyzed the effects of the n−3 fatty acid desaturase on the expression of transcription factors and their genes involved in PUFA synthesis in GEFCs. This research lays a foundation for studying the molecular mechanisms of this gene in the regulation of PUFA metabolism or *fat1*-related signal pathways in the goat, as well as the generation of *fat1* transgenic goats.

2. Materials and methods

The experimental protocol was approved in according with the guide for the care and use of laboratory animals prepared by the Ethics Committee of Nanjing Agricultural University.

2.1. Construction and assessment of the humanized fat1 eukaryotic expression vector

The n−3 fatty acid desaturase cDNA (*fat1* gene) in PCAGGS was kindly provided by Kemian Gou (China Agricultural University, Beijing, China). The *fat1* cDNA of PCAGGS was released by *Eco*R I digestion, and then inserted into the same *Eco*R I site of pEGFP-N1 vector (Invitrogen, USA). The constructs were confirmed by enzymatic digestion and DNA sequencing. The plasmid obtained was named pEGFP-fat1. A schematic representation of this vector was shown in Fig. 1A.

2.2. Isolation and culture of cells

GEFCs were used in this study. They were isolated from the ear of a 1-month-old boer goat. Briefly, the collected tissue samples were cut into 0.5–1 mm^3 and transferred into a 25 cm^2 tissue culture flask. After fixation for 4–6 h, 5 mL Dulbecco's Modified Eagle Media (DMEM; GIBCO, USA) supplemented with 15% fetal bovine serum (FBS; GIBCO, USA) was added to the flask. All cultures were incubated at 37 °C under 5% CO_2 in a humidified atmosphere with media changes every 48 h. When cells reached subconfluency (80–90%), they were subcultured at intervals of 3–5 d by trypsinization using 0.25% trypsin-0.02% EDTA and stored in freezing medium in liquid nitrogen.

2.3. Gene transfection and expression

GEFCs were transfected with pEGFP-fat1 by electroporation according to the manufacturer's protocol. The GEFCs were cultured in fresh growth medium 24 h after transfection. After 48 h of culture, cells were selected by adding 1200 μg/mL of G418 (GIBCO, USA) for 12–14 d, the G418-resistant colonies were detached and reseeded into 12-well culture plates, then grew and proliferated with 600 μg/mL G418, then they were transferred individually into 6-well culture plates until they reached 80% confluency, at which point cells were transferred to 25 cm^2 culture bottles for further expansion prior to screening for the presence of the *fat1* transgene. Approximately 10^5 cells were taken and subjected to PCR. Primers used for the identification of positive cells were 5′-GTGAACGCCAACACCAAGC-3′ (forward) and 5′- CGCCCATGAAGATGTTCCA-3′ (reverse), following denaturation at 94 °C for 5 min, 30 cycles of 30 s at 94 °C, 30 s at 58 °C, 1 min at 72 °C, and a final 7 min extension period at 72 °C. A probe targeting GAPDH gene was used as control. After PCR identification, the positive cells were harvested for Western blot analysis, lipid analysis or qRT-PCR.

2.4. Western blotting

Total cellular protein of GEFCs and T-GEFCs (transfected *fat1* GEFCs) were extracted using RIPA lysis buffer. The total protein content was measured by the BCA method. These proteins were separated using 0.1 g/ml polyacrylamide sodium dodecyl sulfate (SDS) gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes which were then incubated with a blocking buffer (TBST with 0.05 g/mL milk and 0.1% (*v*/*v*) Tween-20, pH 7.5) for 1 h at room temperature, and later, they were incubated overnight at 4 °C with a primary mouse anti-fat1antibody (Abcam, USA, No. ab20163, 1: 800 dilution). β-actin (Santa Cruz, USA, No. SC-47778, 1:2000 dilution) was used as a housekeeping protein control. The treated membranes were washed three times and incubated with the horseradish peroxidase (HRP)-linked secondary antibody diluted (Thermo Pierce, USA, No. 31160, 1: 5000 dilution) in the blocking buffer for 1 h. Then the blots were rinsed three times and detected for immunoreactivity by chemiluminescence detection, and were used to expose X-ray film to visualize immunoreactive signals. Finally, the chemiluminescence intensity of each protein band was quantified using IMAGEJ software (National Institutes of Health, Maryland, USA).

2.5. PUFA analysis

The fatty acid compositions of GEFCs and T-GEFCs were analyzed as described (Yu et al., 2013). Briefly, total cellular lipids (excluding culture media) were extracted with chloroform/methanol (2:1, vol/vol)

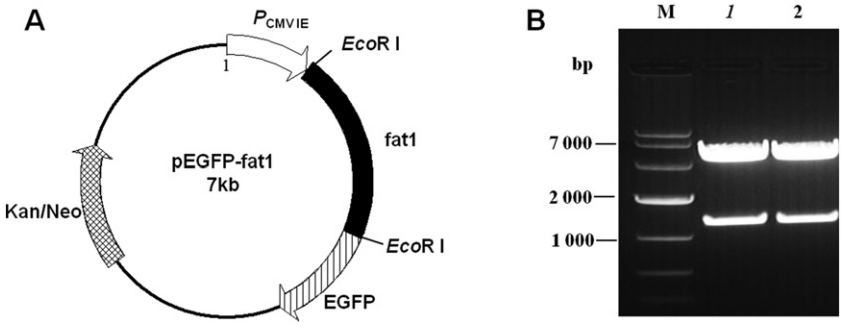

Fig. 1. Construction and assessment of pEGFP-fat1. (A) The *fat1* gene was cloned into pEGFP-N1 (Clontech Laboratories, Inc., Mountain View, CA, USA) which had been digested by *Eco*R I to generate the pEGFP-fat1 vector. (B) Restriction enzymatic digestion of pEGFP-fat1 1 to 2 lane: pEGFP-fat1 digested by *Eco*R I. M: DL10 000 DNA marker.