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Product Fat-1 Transgenic Simmental Crossbred Cattle Endogenously Synthesizing Omega-3 Polyunsaturated Fatty Acids Using OSM

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Abstract: Meat products normally contain low omega-3 fatty acids and high ratio of n-6/n-3 fatty acids may contribute to the prevalence of many diseases. In order to obtained in such beef with high levels of omega-3 fatty acids will be more healthy to meet people's diet, researchers generated the fat-1 transgenic simmental crossbred cattle expressing the *Caenorhabditis elegans c fat-1* gene encoding an omega-3 fatty acid desaturase that converts omega-6 to omega-3 fatty acids and the gene is absent in mammals. In this study, researchers successful product the embryo of fat-1 transgenic simmental crossbred cattle using OSM. The results of Western Blotting and fluoroscopic examination have proved that the *fat-1* gene has been into genome of clone embryo and translated into proteins which could be used to embryo transfer.

Key words: OSM, fat-1, omega-3 fatty acid desaturase, omega-3 fatty acids, embryo, China

INTRODUCTION

Polyunsaturated Fatty Acids (PUFAs) are essential structural components of body. Omega-3 Polyunsaturated Fatty Acids (Omega-3 PUFA) as one importent type of PUFAs are crucial for life (Li-Hong and Xiao-Mei, 2006). The omega-3 PUFAs are important structural components of neuronal membranes and are involved in modulating neurotransmission, cell signaling, gene expression (Rapport, 2003; Kitajka *et al*., 2004) and enzyme activity (Murphy, 1990). Present studies in humans and animals have shown that omega-3 PUFAs have therapeutic use in patients who suffer from arrhythmia (Kang and Leaf, 1996) atherosclerosis (Kris-Etherton *et al*., 2002), hypertension (Howe, 1997) and hyperlipemia (Grimsgaard *et al*., 1997). Docosahexaenoic acid (22:6 n-3; DHA) as one of the most abundant omega-3 Polyunsaturated Fatty Acid (PUFA) is required for normal neuronal development and function (Alessandri *et al*., 2004). Which is a potential factor for treat epilepsy (Schlanger *et al*., 2002; Yuen *et al*., 2005) and neurological disorder characterized by spontaneous and recurrent seizures (Burnham, 2006). Recently studies have suggest that omega-3 PUFAs have a major role to play in inhibiting the growth and evolution of multiple different systemic malignancies. For instance, omega-3 PUFAs could decrease the risk for colorectal cancer (Hall *et al*., 2008) prevent the evolution and development of prostate carcinomas (Mina *et al*., 2008) inducing apoptosis in chemoresistant pancreatic carcinomatous tissue (Hering *et al*., 2007), demonstrate antiproliferative effects in breast carcinomas (Sun *et al*., 2008) and gastric carcinoma (Otto *et al*., 2008).

The omega-3 PUFAs cannot be produced in the body and hence must be supplied in the food. Meat products normally contain small amounts of omega-3 fatty acids and large amounts of omega-6 fatty acids (Simopoulos, 1998; Hlophe and Moyo, 2011). Diets with a high ratio of omega-6/omega-3 fatty acids may contribute to the prevalence of many diseases such as coronary artery disease, cancer, diabetes, arthritis and depression (Simopoulos and Cleland, 2003). However, some lower life such as the roundworm *Caenorhabditis elegans c* harbors a gene called *fat-1* (Spychalla *et al*., 1997) which encodes an omega-3 fatty acid desaturase that can introduce a double bond into omega-6 fatty acids at the omega-3 position of their hydrocarbon chains to form omega-3 fatty acids (Kang *et al*., 2001). Therefore, it is imperative to develop a transgenic cattle that carry *fat-1* gene from *Caenorhabditis elegans c* so that obtained in such beef with high levels of omega-3 fatty acids will be more healthy to meet people's diet. Earlier research in transgenic mice has suggested the feasibility of creating fat-1 transgenic livestock capable of producing omega-3 fatty acids from the corresponding omega-6 fatty acids.

In this study, One-Step Micromanipulation (OSM) has been used to generate the transgenic cattle. Which

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one is a modified approach to SCNT successfully used in the rat (Campbell, 2003) along with limited progress in the monkey (Mitalipov *et al*., 2002; Simerly and Navara, 2003) another species that has been difficult to clone. The process of SCNT involves placing a donor cell in the perivitelline space of a cytoplast and fusing donor and recipient cells with electrical pulses whereas in the OSM, a donor nucleus is isolated and directly injected into the cytoplast. Two protocols that are currently used to produce viable SCNT embryos and OSM was found to be significantly better than the Electrofusion Method. In the study, researchers collected oocytes and ear skin fibroblast cells of simmental crossbred cattle and produce simmental crossbred cattle's viable embryos capable of converting n-6 to n-3 fatty acids with the *fat-1* gene.

MATERIALS AND METHODS

Collection and culture of cumulus-oocyte complexes: Ovaries were collected from prepubertal female simmental crossbred cattle at a local abattoir and transported to the laboratory in 0.9% NaCl solution at 35-39°C. Cumulus-Oocyte Complexes (COCs) were aspirated from antral follicles (2-6 mm in diameter) with an 20-gauge disposable syringe. COCs were washed three times in PBS buffer containing 10% Fetal Bovine Serum (FBS) and 50-60 COCs were transferred to 500 mL of the TCM199 maturation medium containing 20 ug mL^{-1} LH (sigama, USA) and 10 ug mL^{-1} FSH (sigama, USA) that had been covered with mineral oil in a 4-well multidish and equilibrated at 39°C in an atmosphere of 5% CO_2 in air overnight. The oocyte will be matured until being at collection (MII), before SCNT.

Donor cell culture: A small ear skin biopsy was obtained from prepubertal female simmental crossbred cattle at a local abattoir and the tissue was cut into small pieces with fine scissors. The cells were incubated at 37°C in PBS containing 0.05% trypsin and 0.5 mM EDTA overnight and this suspension was centrifuged. The cell pellet was resuspended and cultured in DMEM-F12 medium supplemented with 75 mg mL^{-1} penicillin G, 50 mg mL^{-1} streptomycin and 15% (v:v) Fetal Calf Serum (FCS).

Plasmid construction and transfection: The fat-1 expression vector, PEGFP-N1-fat-1 which contains a fat-1 cDNA and one eGFP sequence driven by the CMV promoter. The *fat-1* gene was clone from *Caenorhabditis elegans c* (contributed by Department of Parasitology, China Agricultural University). Primers for *fat-1* gene were 5'-ATGGTCGCTCATTCCTCAG-3' (forw-ard) and 5'-AGTTATGGCTTTATGCATTCAA-3' (reverse), 5'- CCG GAATTC ATGGTCGCTCATTCCTCAG-3' (forward) and 5'-CGCGGATCC AGTTATGGCTTTATGCATTCAA-3' (reverse). The fibroblasts were transfected by X-fect-mediated (TAKARA, Japen) plasmid PEGFP-N1-fat-1. After G418 selection, surviving cells were used as a nuclear donor.

Spindle removal, nuclear transfer and activation: The OSM technique described previously was employed. Briefly, a fibroblast was aspirated into a 10 μm ID blunt-tip pipette when the cell membrane lysed. The cell was then injected into an oocyte at the side opposite the MII spindle after the injection pipette was passed through the zona and oolemma and then the injection pipette was slowly withdrawn into proximity to the metaphase chromosomes as visualized by micromanipulator. The chromosomes along with a small amount of ooplasm were aspirated into the pipette removed from the oocyte and stained with Hoechst 33342 in a separate drop to confirm spindle removal. About 2 h later, activation was induced by exposure to 5 μM ionomycin for 4 min and then exposure to 2 mM 6-Dimethylaminopurine (DMAP) for 5 h at 37°C in 5% CO_2-balance air. Activated SCNT embryos were cultured in SOFaa culture solution containing 10% FCS at 37°C in 5% CO_2-balance air.

Western blotting: The concentration of proteins was measured by Bradford reagent (Sigma), eparated on 10% SDS-PAGE gels and transferred to Immobilon membranes (Millipore). After blocking in 5% low-fat milk in BST (0.1% Tween 20 in PBS) for 1 h, the membranes were ncubated with GFP Ab-2 antibody (1:500, sigama, USA) horseradish peroxidase labeled sheep anti-rat (1:2000, sigama, USA) overnight at 4°C After washing in PBST, the membranes were incubated in goat anti-rabbit antibody conjuated with horseradish peroxidase (1:5000) for 1 h followed by hree washes in PBST. The signals were detected by Gel imaging system.

Fluoroscopic examination: The fat-1 transfected fibroblast and activated embryos were observed by fluorescence microscope (Nikon, Japen) using objective and excitation wavelengths of CY3 and FITC in order to select the ones carried *eGFP* and *fat-1* gene. For each experiment, the same detector gain, amplifier offset and pinhole parameters were used.

RESULTS AND DISCUSSION

The previous studies have shown that the fat-1 mice have the capable of de novo synthesis of n-3 PUFA from n-6 PUFA. In these studies, the transgenic fat-1

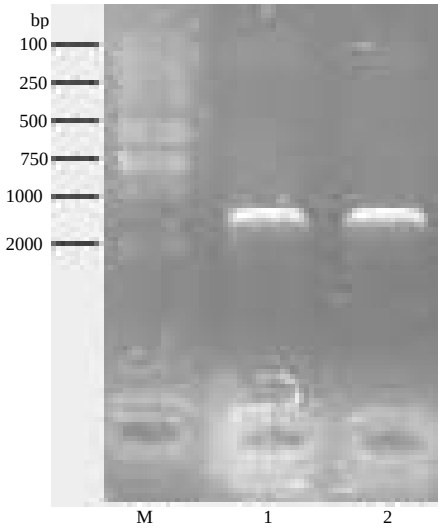

Fig. 1: Polymerase chain reaction amplifications of *C. elegans fat-1*; M: Molecular size markers DL2000 and 1, 2: Amplifications of *C. elegans fat-1*

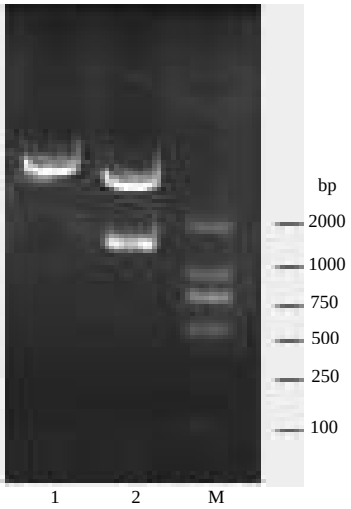

Fig. 2: Restriction map of recombinant plasmid; M: DNA Marker D2000; 1: Double-enzyme digested products and 2: Plasmid control

mouse was as a model to study the pathophysiology of cardiovascular, neurological and psychiatric disorders (Das and Puskas, 2009) to prevent neoplasia (Griffitts *et al*., 2010), to reduction of inflammation and chronic tissue damage (Weylandt *et al*., 2008) and to improve glucose tolerance (Smith *et al*., 2010). Rarely, study aim at providing meat products which are high in low in omega-3 fatty acids. In this study, we successful product the embryo of fat-1 transgenic simmental crossbred cattle using OSM. The complete nucleotide sequence of *Caenorhabditis elegans* c fat-1 cDNA of was amplified (Fig. 1) and which was corrected according to GeneBank (Accession No. NM_001028389). This cDNA sequence was a single open reading frame without any signal peptide and which encodes a putative polypeptide of 454 amino acids (aa) and the molecular mass of polypeptide was 52.912 kDa according to the result of Signal PV3.0 Server analysis.

On the basis of correct sequence result of fat-1 cDNA, researchers inset fat-1 cDNA at the sites of EcoRI and BamHI to construct the eukaryotic expression vector. The recombinant plasmid with the *fat-1* gene was correct by digestion identification (Fig. 2). To determine the expression of fat-1 proteins in fibroblasts, the eGFP was connected to the fat-1 expression vector. Through determine the signal of eGFP and fat-1 proteins can be distinguished in fibroblasts. The fat-1 transfected fibroblasts were subsequently cultured in 250 ug mL^{-1} G418, the survivals were almost all have fluorescence

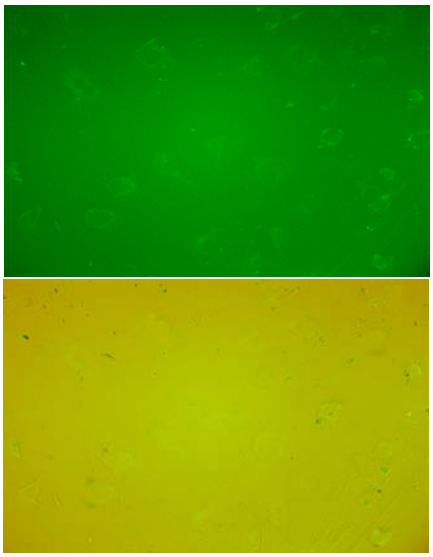

Fig. 3: Transgenic cell clones after selection for 7 days with G418; bright-field pictures of cells on the right; corresponding images under a fluorescence microscope on the left (Wavelengths: CY3 and FITC; magnification: 10x40)

signal. As shown in the (Fig. 3), the signals were detected in cytoplasm and cytomembrane but not in nucleus. What is the same as the previous description (Murphy, 1990). The survivals carried signals were select and been used to Western blotting. By Western blotting, the protein of fat-1 was evaluated by immunoblot analysis using recombinant protein which was 72.9 kDa consisted of a 52.9 kDa of *fat-1* gene protein and 27 kDa of eGFP

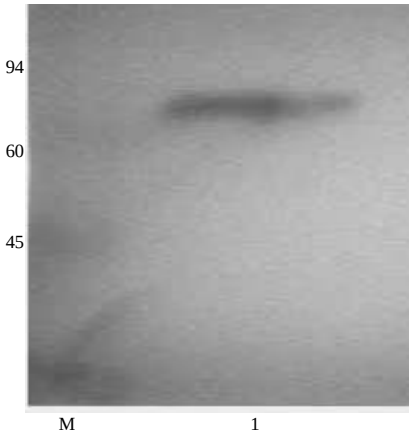

Fig. 4: Western-blotting of expressed recombinant protein; M: Protein marker (Unit: kDa) and 1: Western-blotting of expressed recombinant proteins

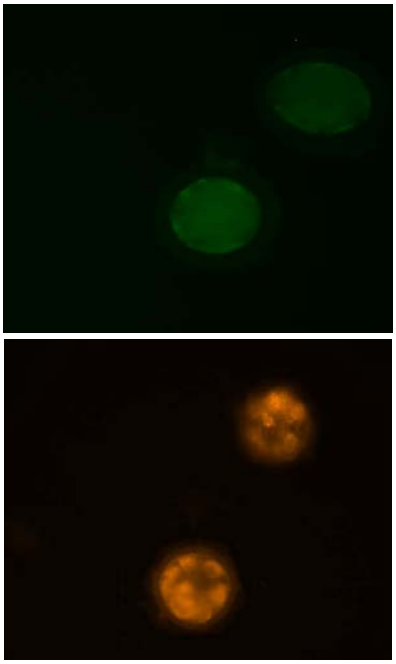

Fig. 5: GFP-positive embryos expressing the transgene from pGFP-N1-fat1 plasmid; the 8-cell embryos under the fluorescence microscope using the wavelengths of RED on the left; corresponding the morulaes using the wavelengths of FICT on the right (magnification: 10×10)

protein (Fig. 4). Researchers could certain this protein was correct corroding to the electrophoretogram. Researchers could be sure that the *fat-1* gene have been into genome of fibroblasts and translated into proteins according to these evidences. The survivals could be used to OSM as donor. In the study, the early embryos which carried the *fat-1* gene have also been examined by fluorescence microscope. Researchers examined three preimplantation stages include two-cells, four-cells and blastocyst. After cleavage, fluorescence signals were characterized by the presence of larger and even more intensely foci in the blastomere of embryos (Fig. 5).

CONCLUSION

The study shows that strong signal were found everywhere in the blastocyst especially in the periphery of blastocyst but there were not any fluorescence signals in transparent zone. So, the blastocyst which carried the *fat-1* gene could be used to embryo transfer and then the generation of cloned cattle will be examining by gas chromatography in order to the level of omega-3 fatty acids from omega-6 analogs in future study. In addition, the fat-1 transgenic simmental crossbred cattles provide a large animal model in which to product such beef with high levels of omega-3 fatty acids will be more healthy to meet people's diet.

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