Electronic Journal of Biotechnology 17 (2014) 224–229

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Construction of a eukaryotic expression vector for pEGFP-FST and its biological activity in duck myoblasts

Xinxin Li, Jiwen Wang *, Hehe Liu, Haohan Wang, Lingli Sun, Chao Yang, Liang Li, Yi Zheng

Institute of Animal Genetics and Breeding, Sichuan Agricultural University, Ya'an 625014, China

ARTICLE INFO

Article history:
Received 9 February 2014
Accepted 30 June 2014
Available online 5 August 2014

Keywords:
Follistatin
Muscle hypertrophy
Overexpression
Transfection efficiency

ABSTRACT

Background: Follistatin (FST), a secreted glycoprotein, is intrinsically linked to muscle hypertrophy. To explore the function of duck FST in myoblast proliferation and differentiation, the pEGFP-FST eukaryotic expression vector was constructed and identified. The biological activities of this vector were analyzed by transfecting pEGFP-FST into cultured duck myoblasts using Lipofectamine™ 2000 and subsequently determining the mRNA expression profiles of FST and myostatin (MSTN).
Results: The duck pEGFP-FST vector was successfully constructed and was confirmed to have high liposome-mediated transfection efficiency in duck myoblasts. Additionally, myoblasts transfected with pEGFP-FST had a higher biological activity. Significantly, the overexpression of FST in these cells significantly inhibited the mRNA expression of MSTN (a target gene that is negatively regulated by FST).
Conclusions: The duck pEGFP-FST vector has been constructed successfully and exhibits biological activity by promoting myoblast proliferation and differentiation in vitro.

1. Introduction

Follistatin (FST), also referred to as FSH inhibiting protein (FSP) [1], is a single chain, glycosylated polypeptide that has an inhibitory effect on follicle-stimulating hormone (FSH). Previous research has demonstrated that FST is expressed in almost all tissues (e.g., kidney, trabecular meshwork and testis [2,3,4]), and that FST possesses extensive physiological functions in these tissues. FST regulates the development and regenerative processes of the kidney, and modulates the production of androgen. The FST gene is considered a candidate gene for the induction of muscle myofiber hypertrophy, and recent research has shown that FST functions in the development of muscle in mice [5]. Previous research has also indicated that FST may promote muscle fiber hypertrophy in a mouse model via activation of satellite cells, causing them to fuse into muscle fibers. For example, both an FST transgene [6], and transfected FST that were delivered by an adeno-associated virus [7], have an effect on satellite cell proliferation and muscle fiber hypertrophy in mice [8]. Additionally, the depletion of FST in mice leads to prenatal lethality associated with impaired muscle development [9]. FST is also known to be a powerful inhibitor of myostatin (MSTN), a negative regulator of muscle development [10]. MSTN knock-out mice displayed a two-fold increase in muscle mass compared with wild-type mice [6], and over-expression of FST in these animals lead to an increase in muscle mass that was four-fold greater than in normal mice [11]. These studies suggest a close relationship between FST and skeletal muscle hypertrophy in mammals. In contrast, the roles of FST in skeletal muscle remain largely uncharacterized in birds.

Peking ducks (*Anas platyrhynchos domestica*) constitute a considerable portion of the poultry meat market. We previously cloned the duck FST coding domain sequence (CDS), and found that the sequence in ducks was different from that in mammals [12]. We also cloned the duck FST gene into a prokaryotic expression vector and purified a duck FST recombinant fusion protein. When administered into adult duck leg muscle tissues, the recombinant FST protein was shown to possess biological activity and promote muscle growth [13]. To better understand the mechanism of FST in regulating muscle hypertrophy in birds, we sought to construct a eukaryotic expression vector for duck FST with biological activity in promoting myoblast proliferation and differentiation.

In the present study, duck FST cDNA was inserted into the eukaryotic expression vector pEGFP-N1 to generate pEGFP-FST, which was then transfected into duck myoblasts where it exhibited some biological activities. These results provide technical support for basic research on the regulation of FST in skeletal muscle

* Corresponding author.
E-mail address: wjw2886166@163.com (J. Wang).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.ejbt.2014.07.002

hypertrophy, and therefore elucidate potential future studies of this subject.

2. Materials and methods

2.1. Animals

Peking duck eggs at 13 d of incubation were obtained randomly from the Sichuan Agricultural University Waterfowl Breeding Experimental Farm. All of the eggs were incubated under the same conditions at a temperature of 37 ± 0.5°C and a humidity of 86–87%.

2.2. Construction of duck pMD-19T-FST and pEGFP-FST

Based on the total sequence length of the duck FST CDS [12], a pair of primers was designed: forward 5′ TTGATATCGGGGACTGCTGGCTCCGG CAG 3′; and reverse 5′ GGCTCGAGTTACC ACTCTAGAATGGAA 3′. The following PCR amplification cycles were performed: 5 min at 95°C for an initial denaturation, 34 cycles of 30 s at 95°C, 30 s at 51°C for primer annealing, an extension time of 60 s at 72°C, and 10 min at 72°C for the final extension. The PCR products were electrophoresed in 1.5% (w/v) agarose gels and stained with ethidium bromide. Next, the PCR products were purified and recovered using an agarose gel extraction kit (Watson Biomedical Inc., Shanghai, China). The purified FST fragments were ligated to pMD-19T vector (Takara, Japan) at a 9:1 ratio for 1 h at 16°C. For amplification, 10 μL of pMD-19T-FST solution was transformed into 50 μL *E. coli DH5a* cells, with the specific steps as follows: incubation on ice (-6°C) for 30 min, and heat stress (42°C) for 45 s, cold stress (-6°C) for 1 min. Positive clones were isolated and shaken in a thermostatic culture cradle overnight at 37°C, and a random analysis of 20 clones was then conducted using PCR. Finally, sequencing analysis was conducted by Invitrogen Life Technologies.

Based on restriction enzyme mapping of the CDS fragments of duck FST and the multiple cloning sites present in the pEGFP-N1 vector (Clontech, CA, USA), *Xho I* and *EcoRI* were chosen as the insertion sites. A pair of primers was designed representing the two ends of the FST CDS, and an *XhoI* restriction enzyme site was inserted upstream of the FST CDS. The forward primer was designed as follows: 5′ CTCTCG AGTTAAATCAGAGGATCCA 3′ (where CTCGAG is the *XhoI* site). The reverse primer was designed as follows: 5′ CGGAATTCTTACCACTCTAG AATGG 3′ (where GAATTC is the *EcoRI* site). The FST CDS should be in the same reading frame as the downstream EGFP gene sequence to ensure co-expression of the fusion protein.

To improve the amplification efficiency, the CDS of the FST gene was amplified using the following PCR cycles: 4 min at 95°C for initial denaturation, 34 cycles of 45 s at 95°C, 40 s at 52°C for primer annealing, an extension time of 60 s at 72°C, and 10 min at 72°C for a final extension. The PCR product was recovered and cloned into the pMD-19T simple vector, and was then transformed into competent *DH5α* cells. Positive clones were isolated and shaken overnight at 37°C. Plasmids were extracted from sense colonies using the TIANprep Mini Plasmid Kit (Tiangen, Beijing, China) and digested using *XhoI* and *EcoRI* (Takara). The reaction mixture was as follows: 4 μL of the FST gene, 2.5 μL (10×) T4 DNA ligase buffer, 1 μL pEGFP-N1, 1 μL of T4 DNA ligase (NEB, USA) and 16.5 μL of sterilized water. A cDNA fragment of 1032 bp was recovered and directly ligated into a pEGFP-N1 eukaryotic expression vector that had been digested with *XhoI* and *EcoRI*, and then transformed into competent *DH5α* cells. Positive clones were isolated and shaken overnight at 37°C and confirmed via sequencing by Invitrogen Life Technologies. The identification results for pMD-19T-FST and pEGFP-FST, as well as a map of the final recombinant plasmid (pEGFP-FST), are shown in Fig. 1.

2.3. Cell culture

Primary duck myoblast cultures were prepared according to the method described by Liu et al. [14]. Myoblasts from 13-d-old eggs were isolated based on a differential attachment and were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). The cells were maintained in 5% CO_2 at 37°C. When confluent, the cells were transferred to a 6-well plate using a split ratio of 1:2.

2.4. pEGFP-FST transfection

Duck myoblasts were transfected with pEGFP-FST when the cells reached 70% confluency. The cells were divided into three groups: pEGFP-FST, pEGFP-N1 and control. Transfection was carried out using Lipofectamine™ 2000 (Beyotime, Shanghai) according to the manufacturer's instructions. In each well, cells were transfected with the following liposomal transfection mixture: 12.5 μL (2.5 μg) of DNA, 47.5 μL of DMEM and 15 μL of liposomes. After 12 and 24 h, the cells were collected to conduct subsequent assays. All experiments were performed in triplicate.

2.5. Analysis of transfection efficiency

After 24 h, the expression of EGFP in myoblasts was observed under a fluorescence microscope (Nikon TE2000, Japan), and the number of cells in every well exhibiting positive EGFP expression was counted (Fig. 2).

2.6. MTT assay and morphological observation

Myoblast viability was determined based on the amount of MTT reduced to formazan. After transfection with either pEGFP-FST or pEGFP-N1, culture medium containing 0.5 mg/mL MTT was added to each well and the cells were incubated at 37°C for 3 h, at which point DMSO was added to dissolve the formazan crystals. The absorbance at 570 nm was then measured. Twenty-four hours after transfection, changes in cell morphology were observed and the number of myoblasts in the three groups was recorded (Fig. 3).

2.7. Real-time PCR analysis

Total RNA was isolated from duck myoblasts using the Trizol reagent (Takara, Dalian, China), and the concentration of each RNA sample was determined using a NanoVue Plus spectrophotometer (GE Healthcare Bio-Sciences AB, Sweden). All RNA samples were subsequently adjusted to the same concentration. A SYBR Prime Script RT-PCR Kit (TaKaRa, Dalian, China) was then used for reverse transcription-PCR (RT-PCR) according to the manufacturer's protocol. The relative mRNA expression of FST and MSTN was analyzed by real-time PCR using the IQ™5 System (Bio-Rad, USA) with *β-actin* (Genbank No: EF667345.1) and *GAPDH* (Genbank No: GU564233.1) serving as reference genes. The primer information is listed in Table 1. The PCR reactions were carried out in a 96-well plate in a 25 μL reaction volume. Each reaction mixture contained 12.5 μL of SYBR® Green I PCR Master Mix (Takara, Japan), 2.5 μL of normalized template DNA, 0.5 μL of each primer and 9.5 μL of sterile ultrapure water. The relative expression of FST and MSTN was calculated using the "normalized relative quantification" method followed by $2^{-\triangle\triangle Ct}$. PCR reactions were performed in triplicate for each sample.

2.8. Statistical analysis

The real-time PCR data were subjected to analysis of variance (ANOVA), and the means were compared for significance using