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''CONSTRUCTION OF A EUKARYOTIC EXPRESSION VECTOR FOR PEGFP-FST AND ITS BIOLOGICAL ACTIVITY IN DUCK MYOBLASTS''

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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 possess high liposome-mediated transfection efficiency in duck myoblasts. Additionally, myoblasts transfected with pEGFP-FST had a better biological activity. Significantly, the overexpression of FST in these cells significantly inhibited the mRNA expression of MSTN (a target gene that's 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.

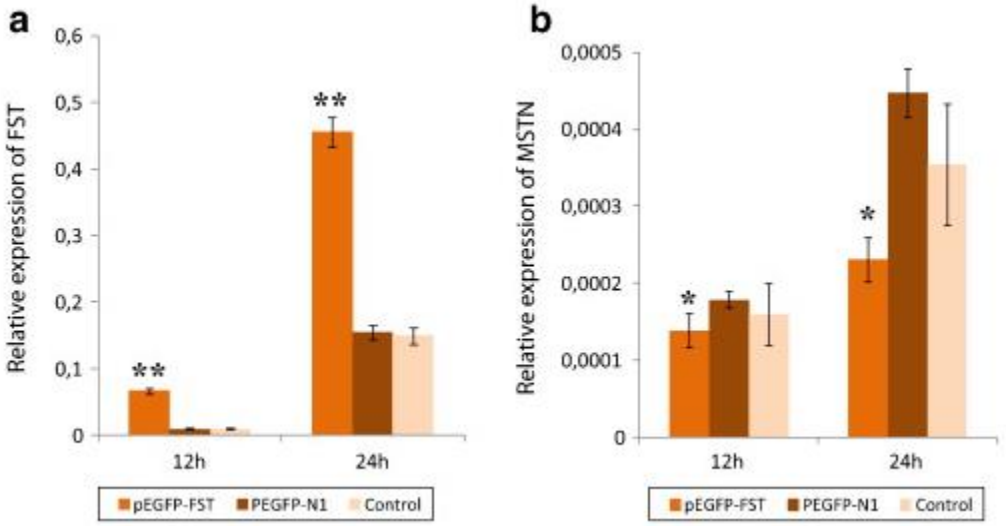

Fig. 4. Effect of pEGFP-FST transfection on the mRNA expression of FST and MSTN after 12 and 24 h. (a) The relative expression of FST after transfection for 12 and 24 h. (b) The relative expression of MSTN after transfection for 12 and 24 h. Duck *β-actin* (EF667345) and *GADPH* (AY436595) were used as the internal controls. The label"**"above the bar indicates $P < 0.01$, and "*"above the bar indicates $P < 0.05$.

FollistatinMuscle hypertrophyOverexpressionTransfection efficiency

1. Introduction

Follistatin (FST), also brought up as FSH inhibiting protein (FSP) [1], may be a single chain, the glycosylated polypeptide that has an inhibitory effect on gonadotropin (FSH). Previous research has demonstrated that FST is expressed in the majority tissues (e.g., kidney, trabecular meshwork, and testis [2,3,4]), which FST possesses extensive physiological functions in these tissues. FST regulates the event and regenerative processes of the kidney and modulates the assembly of androgen. The FST gene is taken into account a candidate gene for the induction of muscle myofiber hypertrophy, and up to date research has shown that FST functions within the development of muscle in mice [5]. Previous research has also indicated that FST may promote somatic cell hypertrophy during a mouse model via activation of satellite cells, causing them to fuse into muscle fibers. as an example, both an FST transgene [6], and transfected FST

that was delivered by an adeno-associated virus [7], have an impression on satellite cell proliferation and muscle fibre hypertrophy in mice [8]. Additionally, the depletion of FST in mice ends up in prenatal lethality related to impaired muscle development [9]. FST is additionally known to be a strong 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 ends up in a rise in muscle mass that was four-fold greater than in normal mice [11]. These studies suggest a detailed relationship between FST and muscle hypertrophy in mammals. In contrast, the roles of FST in muscle remain largely uncharacterized in birds.

Peking ducks (Anas platyrhynchos domestica) constitute a substantial portion of the poultry store. We previously cloned the duck FST coding domain sequence (CDS), and located 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 the biological activity and promote muscle growth [13]. to higher 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 get 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 musculus hypertrophy and thus 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 Agri-

cultural University Waterfowl Breeding Experimental Farm. All of the eggs were incubated under the identical 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 entire sequence length of the duck FST CDS [12], a pair of primers was designed: forward 5′ TTGATATCGGGGACTGCTGGCTCCGGCAG 3′; and reverse 5′ GGCTCGAGTTACC ACTCTAGAATGGAA 3′. the subsequent 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 ultimate 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 the pMD-19T-FST solution was transformed into 50 μL E. coli DH5a cells, with the precise steps as follows: incubation on ice (-6°C) for 30 min, and warmth stress (42°C) for 45 s, cold stress (-6°C) for 1 min. Positive clones were isolated and shaken during a thermostatic culture cradle overnight at 37°C, and random analysis of 20 clones was then conducted using PCR. Finally, sequencing analysis was conducted by Invitrogen Life Technologies.

Based on restriction endonuclease mapping of the CDS fragments of duck FST and therefore the multiple cloning sites present within the pEGFP-N1 vector (Clontech, CA, USA), Xho I and EcoRI were chosen because the insertion sites. A pair of primers was designed representing the 2 ends of the FST CDS, and an XhoI restriction nuclease site was inserted upstream of the FST CDS. The forward primer was designed as follows: 5′ CTCTCGAGTTAAATCAGAGGATCCA 3′ (where CTC GAG is that the XhoI site). The reverse primer was designed as follows: 5′ CGGAATTCTTACCACTCTAGAATGG 3′ (where GAATTC is that the EcoRI site). The FST CDS should be within the same reading frame because the downstream EGFP gene sequence to confirm co-expression of

the fusion protein.

To improve the amplification efficiency, the CDS of the FST gene was amplified using the subsequent 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, so 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, also as a map of the ultimate recombinant plasmid (pEGFP-FST), are shown in Fig. 1.

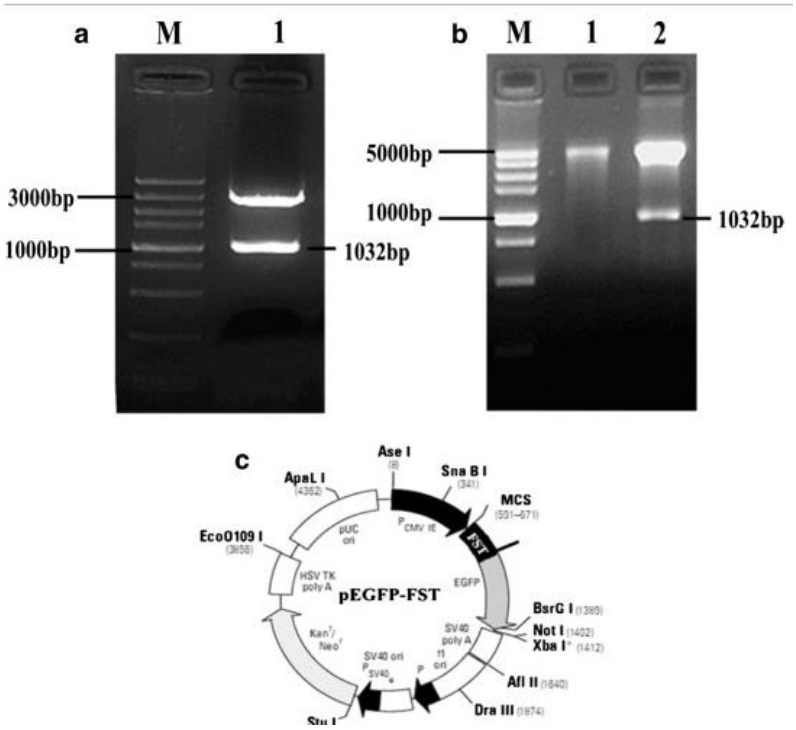

Fig. 1. Identification of duck pMD-19T-FST and pEGFP-FST. (a) Identification of the recombinant plasmid pMD-19T-FST by enzyme digestion (*XhoI* and *EcoRI*) and 1.5% agarose gel electrophoresis after a PCR reaction. M: DL5000 Marker; 1: plasmid pMD-19T-FST digested with *EcoRI* and *XhoI*. (b) Digestion of pEGFP-FST with *XhoI* and *EcoRI*. M: DL5000Marker; 1: pEGFP-N1; 2: pEGFP-FST recombinant plasmid enzyme digestion bands. (c) The map of the final cloned recombinant plasmid (pEGFP-FST).

2.3. Cell culture

Primary duck myoblast cultures were prepared per the strategy described by Liu et al. [14]. Myoblasts from 13-d-old eggs were isolated supported 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% CO2 at 37°C. When confluent, the cells were transferred to a 6-well plate employing a split ratio of 1:2.

2.4. GFP-FST transfection

Duck myoblasts were transfected with pEGFP-FST when the cells reached 70% conflu-

ency. The cells were divided into three groups: pEGFP-FST, pEGFP-N1, and control. Transfection was administered using Lipofectamine™ 2000 (Beyotime, Shanghai) in keeping with the manufacturer's instructions. In each well, cells were transfected with the subsequent 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 also the number of cells in every well exhibiting positive EGFP expression was counted (Fig. 2).

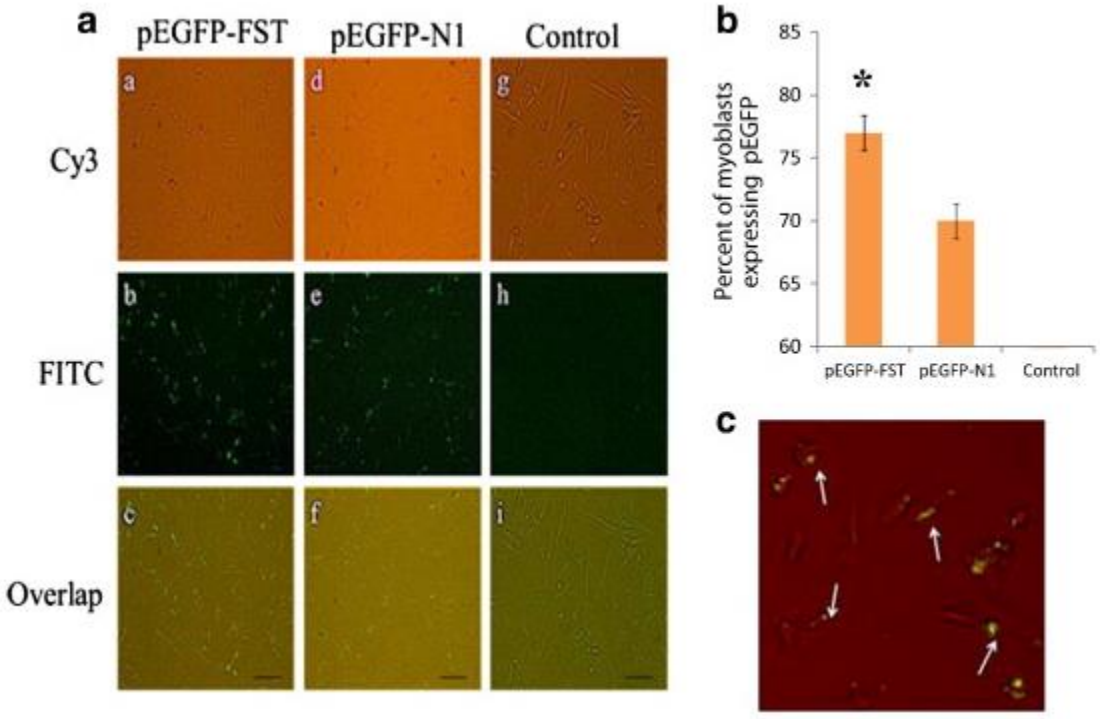

Fig. 2. Lipofection efficiency of myoblasts (fluorescence images taken 24 h post-transfection). (a) The expression of EGFP in duck myoblasts (× 100). Note: a, b and c show pEGFP-FST transfected myoblasts (transfection); d, e and f show pEGFP-N1-transfected myoblasts; and g, h and i show the control. Comparing by rows, a, d and g show the shape of myoblasts (Cy3); b, e and h reflect the green fluorescent protein (FITC); and c, f and i show the overlap of the two images. The cells containing EGFP were identified as positive cells. (b) This figure shows that the highest efficiency of transfection was obtained with the pEGFP-FST group and the pEGFP-N1 group. The efficiencies were 77% and 70%, respectively. The number of myoblasts containing EGFP protein was counted and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD). The arrows in (c) indicate the expression of green fluorescent EGFP.

2.6. MTT assay and morphological observation

Myoblast viability determined supported the quantity of MTT reduced to formazan. After transfection with either pEGFP-FST or pEGFP-N1, a substance containing 0.5 mg/mL MTT was added to every well and therefore 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. day after transfection, changes in cell morphology were observed and also the number of myoblasts within the three groups was recorded (Fig. 3).

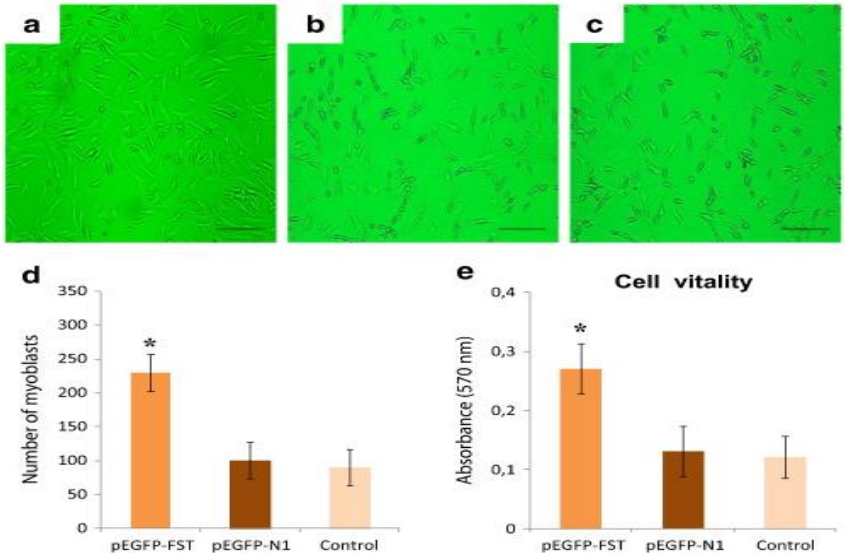

Fig. 3. Effect of pEGFP-FST transfection on myoblast morphology and vitality in vitro (× 100). (a) pEGFP-FST group, B: pEGFP-N1 group, C: Control group, D: The number of myoblasts in the pEGFP-FST group, the pEGFP-N1 group and the control group. The number of myoblasts was counted and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD). E: Vitality of duck myoblasts after transfection with pEGFP-FST for 24 h. The results are presented as the mean ± SEM (n = 3). Data were analyzed by ANOVA and Tukey's test.

2.7. Real-time PCR analysis

Total RNA was isolated from duck myoblasts using the Trizol reagent (Takara, Dalian, China), and also the concentration of every RNA sample resolve employing a NanoVue Plus spectrophotometer (GE Healthcare Bio-Sciences AB, Sweden). All RNA samples were subsequently adjusted to the identical concentration. A SYBR Prime Script RT-PCR Kit (TaKaRa, Dalian, China) was then used for reverse transcription-PCR (RT-PCR) consistent with 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 distributed during a 96-well plate in an exceedingly 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 every 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-ΔΔCt. PCR reactions were performed in triplicate for every sample.

Table 1. Primers used in this study.

Gene	Primer sequence (5′–3′)	Product length (bp)	Annealing temperature (°C)
FST			
F	ACAACTTACCCAAGCGAGTGTG	145	58
R	CATCTTCCTCTTCTTCCTCTGG		
MSTN			
F	GCACTGGTATTTGGCAGAGTATT	142	60
R	TCACCTGGTCCTGGGAAAGT		
β-actin			
F	GCTATGTCGCCCTGGATTTC	168	55
R	CACAGGACTCCATACCCAAGAA		
GAPDH			
F	AAGGCTGAGAATGGGAAAC	254	50
R	TTCAGGGACTTGTCATACTTC		

Note: F, R — forward and reverse primers, respectively.

2.8. Statistical analysis

The real-time PCR data were subjected to analysis of variance (ANOVA), and also the means were compared for significance using Tukey's test, performed by SAS (SAS

Institute, Cary, NC, USA). A p-value of but 0.05 was considered to be statistically significant.

3. Results

3.1. Identification of duck pMD-19T-FST and pEGFP-FST

Complete digestion of the pMD-19T-FST vector with XhoI and EcoRI produced the expected fragments (Fig. 1a).

The target gene fragment was successfully ligated to the 5′ end of the EGFP cDNA, guaranteeing that the CDS of FST was within the same reading frame as EGFP. the anticipated 1032 bp fragment was obtained by complete digestion of the recombinant pEGFP-FST plasmid with XhoI and EcoRI (Fig. 1b).

3.2. Transfection efficiency analysis of the duck expression vector pEGFP-FST in vitro

The expression of the EGFP reporter gene was observed using microscopy (Nikon, Japan) 24 h after transfection (Fig. 2). The results showed that, in both the pEGFP-FST group and therefore the pEGFP-N1 group, large numbers of myoblasts expressed green fluorescent protein. EGFP was expressed in 77% of the cells within the pEGFP-FST group and 70% of the cells within the pEGFP-N1 group (Fig. 2b), suggesting that both pEGFP-FST and pEGFP-N1 is effectively transfected into myoblasts, leading to high levels of EGFP expression.

3.3. Effect of pEGFP-FST transfection on myoblast morphology and vitality in vitro

Myoblast cell counts were significantly increased after pEGFP-FST was successfully transfected into duck myoblasts, with the bulk of cells forming myotubes by fusion as shown in Fig. 3a–c. The numbers of myoblasts in both the pEGFP-N1 and controls group were significantly below within the pEGFP-FST group, with only some cells fusing into myotubes, as shown in Fig. 3d ($P < 0.05$). there have been no obvious differences

in either the amount of myoblasts or myoblast morphology between the pEGFP-N1 group and also the control group. These results suggested that transfection using Lipofectamine™ 2000 wasn't toxic to the cells which FST could promote the proliferation and differentiation of myoblasts. Additionally, myoblast viability increased significantly within the pEGFP-FST group, as demonstrated by the MTT assay. As shown in Fig. 3e, the pEGFP-FST group produced a better OD value than the pEGFP-N1 group or the control group ($P < 0.05$). Collectively, these results indicate that pEGFP-FST increases myoblast viability and significantly promotes the proliferation and differentiation of myoblasts.

3.4. Effect of pEGFP-FST transfection on the mRNA expression of FST and MSTN

As shown in Fig. 4a, FST mRNA expression was significantly higher within the pEGFP-FST group as compared to the pEGFP-N1 or control groups ($P < 0.01$), suggesting that FST was successfully transfected into myoblasts and efficiently expressed. day post-transfection, the expression of FST in myoblasts was beyond at 12 h, indicating that the transfection efficiency increased over time. As shown in Fig. 4b, MSTN mRNA expression within the pEGFP-FST group was significantly not up to that within the pEGFP-N1 or control groups ($P < 0.05$). Nevertheless, the expression of MSTN increased over time, indicating that the expression of MSTN also occurred in a very time-dependent manner. Taken together, these results demonstrate that the over-expression of FST inhibits the expression of MSTN.

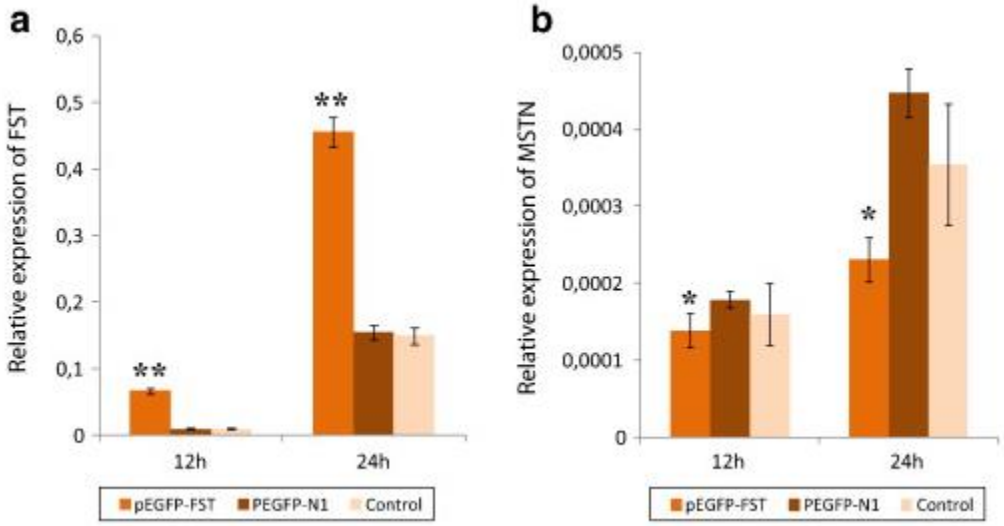

Fig. 4. Effect of pEGFP-FST transfection on the mRNA expression of FST and MSTN after 12 and 24 h. (a) The relative expression of FST after transfection for 12 and 24 h. (b) The relative expression of MSTN after transfection for 12 and 24 h. Duck *β-actin* (EF667345) and *GADPH* (AY436595) were used as the internal controls. The label"**"above the bar indicates $P < 0.01$, and "*"above the bar indicates $P < 0.05$.

4. Discussion

The eukaryotic expression system is a good thanks to explore the functions of latest genes in vitro and might be employed in both the medical and agricultural fields. The eukaryotic expression vector pEGFP-C1-BMP-2 was originally generated and transfected into COS-7 cells to explore the function of BMP in bone and cartilage development [15]. Since the pEGFP vector carries the EGFP gene, any expressed fusion proteins from the pEGFP plasmid will contain both the target protein and also the EGFP protein. Additionally, the pEGFP plasmid has been demonstrated to possess no toxic effects on cells [16]. Therefore, to check the effect of duck FST on myoblasts and to get the inspiration for a radical study of the role of FST in duck muscle development, we generated a pEGFP-FST eukaryotic expression vector.

To investigate the biological activity of pEGFP-FST in myoblasts, the pEGFP-FST eukaryotic vector was inserted into duck myoblasts via liposome-mediated transfection. The results showed that pEGFP-FST possessed specific biological activities. The trans-

fection efficiency of pEGFP-FST was significantly on top of that of either pEGFP-N1 or the control vector, and pEGFP-FST enhanced duck myoblast viability while mildly promoting myoblast proliferation and differentiation. day post-transfection, pEGFP-FST displayed high transfection efficiency (77%) that was in step with the previous research showing a high transfection efficiency for the pIRES2-EGFP-myf6 vector in bovine myoblasts [17]. However, further study is required to see the underlying mechanisms by which FST affects duck myoblast proliferation and differentiation.

FST may be a secreted glycoprotein that promotes muscle hypertrophy. Previous research has shown that FST is capable of inducing muscle hypertrophy by activating satellite cells and influencing the expression of myogenesis-related genes [13]. Additionally, inhibition of MSTN by FST could be a recently identified novel mechanism for the promotion of muscle hypertrophy [8]. MSTN may be a strong negative regulator of musculus mass. In mammals, FST can inhibit the binding of the C-terminus of the MSTN dimer to the ActRIIB receptor in transfected COS cells [6]. employing a two-hybrid analysis, it absolutely was also shown that FST and mature MSTN form a fancy in both yeast and mammalian cells [10]. Additionally, systemic administration of FST in mice inhibits the wasting effect of MSTN in vivo [18]. In our research, FST mRNA expression increased significantly after transfection with pEGFP-FST, with higher expression of FST at 24 h than at 12 h. Our research is in line with that of Sun et al. [17], who also showed that the expression of pEGFP-MyoD in myoblasts is higher at 24 h than 12 h and 36 h. to work out the role of FST in duck myoblasts, likewise on further validate the effect of FST over-expression on MSTN, we examined their expression levels of both genes in pEGFP-FST, pEGFP-N1, and control myoblasts at two stages (Fig. 4b). The results showed that the expression levels of MSTN were significantly lower within the pEGFP-FST group than within the other two groups at both stages which the expression of MSTN within the 24 h group was above that within the 12 h group. the extent of MSTN expression failed to change remarkably within the pEGFP-N1 or the control group, indicating that the over-expression of FST significantly inhibited the expression of MSTN. In duck myoblasts, transfected pEGFP-FST may function an inhibitor to

down-regulate the expression of MSTN. Moreover, the results demonstrated that the mechanism by which FST inhibits MSTN within the duck was like what has been previously reported in mammals, and also showed that duck pEGFP-FST possessed specific biological activity leading to the inhibition of MSTN mRNA expression in myoblasts. Thus, these results further demonstrate the successful construction of pEGFP-FST.

Taken together, these results suggest that the duck pEGFP-FST plasmid has been successfully constructed which it demonstrates biological activity by promoting myoblast proliferation and differentiation in vitro. Our preliminary studies provide the groundwork for further research on the roles of FST in duck myoblasts and pave the way for future studies of the intracellular downstream signaling mechanisms liable for FST's ability to market duck muscle development.

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