ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
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OPEN ACCESS International Journal of Poultry Science

ISSN 1682-8356
DOI: 10.3923/ijps.2018.8.21

Research Article
Cloning of Japanese Quail (*Coturnix japonica*) Follistatin and Production of Bioactive Quail Follistatin288 in *Escherichia coli*

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Abstract

Background and Objective: Follistatin (FST) is a cysteine-rich autocrine glycoprotein and plays an important role in mammalian prenatal and postnatal development. The FST binds to and inhibits myostatin (MSTN), a potent negative regulator of skeletal muscle growth, thus FST abundance enhances muscle growth in animals. The objective of this study was to determine cDNA sequence of quail FST and to produce biologically active quail FST288 (qFST288) in an *Escherichia coli* (*E. coli*) expression system. **Materials and Methods:** Total RNA isolated from quail ovary tissue was used in performing 3'-and 5'-RACE to determine the full-length mRNA sequence of quail FST. The full-length quail FST cDNA consisted of 1118 bp with an open reading frame (ORF) of 1032 bp. The qFST amino acid sequence deduced from qFST cDNA was identical to chicken FST except the sequence at 28 position. To produce recombinant qFST288 protein, Gibson assembly cloning method was used to insert the DNA fragments of qFST288 into pMALc5x vector downstream of the maltose-binding protein (MBP) gene and the plasmids containing the inserts were eventually transformed into shuffle *E. coli* strain for protein expression. **Results:** Soluble expression of the qFST288 protein was achieved through the experiments and the protein could be easily purified by the combination of amylose and heparin resin affinity chromatography. In an *in vitro* reporter gene assay, MBP-qFST288 demonstrated its capacity to suppress the activities of MSTN or activin A. **Conclusion:** Through cloning of quail FST cDNA, it was discovered that amino acid sequence of quail FST is identical to that of chicken FST. In addition, it was demonstrated that bioactive qFST288 could be produced in *E. coli*.

Key words: Quail follistatin, myostatin, cloning of quail, meat-animal, muscle growth

Received: October 25, 2017 Accepted: November 17, 2017 Published: December 15, 2017

Citation: Yusuf Mohammed Maaeni, Sang Beum Lee, Dong Hyuck Choi, Paul E. Mozdziak and Yong Soo Kim, 2018. Cloning of Japanese quail (*Coturnix japonica*) follistatin and production of bioactive quail follistatin288 in *Escherichia coli*. Int. J. Poult. Sci., 17: 8-21.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Muscle is an economically important tissue for meat-producing animals because as a main component of meat, it provides humans with high quality proteins as well as B vitamins, Fe and Zn. In this regard, improving the efficiency of meat-animal production is crucial to a sustainable supply of quality proteins to increasing human population with minimal environmental impacts. Some studies have indicated that enhancing skeletal muscle growth improves the efficiency of meat-animal production via improvement in feed utilization[1].

Myostatin (MSTN), also called as GDF-8, is a member of the TGF-beta superfamily and acts as a negative regulator of skeletal muscle development and growth[2,3]. In lab animals, inhibiting MSTN activity has shown to enhance skeletal muscle growth in normal conditions or ameliorate muscle wasting in atrophic disease[4-8]. In broiler, it has been shown that *in ovo* administration of anti-MSTN antibody significantly improved post-hatch skeletal muscle growth[9], illustrating the potential of inhibition of MSTN as a strategy to enhance muscle growth in meat-producing animals. MSTN binds to activin type IIB (ActRIIB) receptor to initiate its signaling cascade leading to muscle mass regulation[6] and few proteins, such as MSTN propeptide, follistatin-related protein 3 (FSTL3) and GDF associated serum protein-1 (GASP-1), have shown to complex with MSTN and suppress its binding to its receptor[10,11]. In addition to these molecules, follistatin (FST), a cysteine-rich autocrine glycoprotein, have shown to bind to MSTN and inhibit MSTN activity *in vitro*[6]. The abundance of FST or FST fragment in muscle via transgenesis, injection of expression plasmid, or single administration of FST gene via adeno-associated virus delivery system significantly increases skeletal muscle mass/strength[6,12-15]. Transgenic rainbow trout overexpressing FST exhibited dramatic muscularity[16]. These data together indicate that FST would be a potential agent to improve skeletal muscle growth of agricultural animals, as well as, to treat skeletal muscle atrophic disorders in humans.

In bird, chicken FST cDNA was sequenced and its expression was investigated[17], but limited information is available on FST cDNA of Japanese quail (*Coturnix japonica*), which belongs to the same order (Galliformes) and family (Phasianidae) as chicken. Japanese quail is easily managed, fast growing, small in size and can produce eggs at a high rate, thus the animal provide stable source of animal proteins in developing countries. Information on FST cDNA sequence of Japanese quail would potentially contribute to improving skeletal muscle growth of Japanese quail. Therefore, the objective of this study was to determine the full-length cDNA of Japanese quail FST and to produce bioactive quail FST288 in *E. coli* to examine its future potential in quail production. The determination of quail cDNA sequence revealed that the amino acid sequence of quail FST was identical to that of chicken FST. These results also show that bioactive, quail FST288 can be produced in an *E. coli* system.

MATERIALS AND METHODS

Total RNA extraction and cDNA synthesis: Ovary and muscle tissue samples from a young, female Japanese quail (*Coturnix japonica*) were homogenized separately in TRIzol reagent (Invitrogen, NY, USA) and total RNA was isolated following the manufacture's protocol. Reverse transcription of the RNA was performed using the M-MLV reverse transcriptase (Invitrogen, NY, USA) and the reaction mixture was stored at-20°C for later use.

Cloning of quail follistatin (qFST) cDNA: For PCR amplification of partial qFST cDNA, a forward (F1) and reverse (R1) primer set was designed by selecting highly conserved region of FST cDNA of various avian species, including *Gallus gallus* (GenBank accession number, NM 205201), *Falco cherrug* (GenBank accession number, XM005443919), *Taeniopygia guttata* (GenBank accession number, XM002197254.2) and *Zonotrichia albicollis* (GenBank accession number, XM-0055490664) (supplementary file 1 shows clustal analysis result). The sequences of F1 and R1 were 5'GGCATGCTCGTACTCCTGAT3' and 5'AGGAAAGCTGTAGT CCTGGTCT3', respectively. This primer set was expected to amplify a PCR product of 983 bp, which corresponded to 25-1008 of the chicken-equivalent FST cDNA ORF sequence. Amplification of reverse-transcribed quail ovary RNA with the primer set resulted in a single PCR product of smaller than 1,000 bp, but not with the reverse-transcribed quail skeletal muscle RNA. Since the size of amplified product from ovary RNA was close to the expected size, the amplified product was excised from the gel, purified and inserted into pGEM-T Easy vector (Promega, WI, USA), followed by validation of insertion into the pGEM-T Easy vector using colony PCR. After validation of insertion, the sequence of inserted cDNA was analyzed and aligned against chicken sequence. The alignment revealed more than 95% homology between the two sequences (supplementary file 2).

The 3-end of cDNA, including poly-A tail, was cloned by the RNA Ligase Mediated Rapid Amplification of cDNA Ends method (First Choice® RLM-RACE Kit, Life Technologies, NY, USA) according to the manufacturer's instruction, using quail

Table 1: Primer sets used in the determination of quail FST full-length cDNA sequence

Primer	Sequence	Region*
F1	5'-GGCATGCTCGTACTCCTGAT-3'	25-44 bp
F2	5'-ACCTCAGCAAAGAGGAGTGC-3'	143-162 bp
F3	5'-CATCCCGTGCAAAGAAAC-3'	261-278 bp
F4	5'-TGTGGGATTTTAAGGTTGGC-3'	767-786 bp
F5	5'-ATGTTAAATCAGAGGATCCA-3'	1-20 bp
F6	5'-GGGAACTGCTGGCTCCGGCA-3'	85-104 bp
R1	5'-AGGAAAGCTGTAGTCCTGGTCT-3'	987-1008 bp
R2	5'-ACATTTCTTCCCGGGTCCAC-3'	296-315 bp
R3	5'-CTCGTAGGCTAATCCAATG-3'	696-715 bp
R5	5'-TTACCACTCTAGAATGGAAG-3'	1013-1032 bp
5'-RACE outer	5'-GCTGATGGCGATGAATGAACACTG-3'	
5'-RACE inner	5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3'	
3'-RACE outer	5'-GCGAGCACAGAATTAATACGACT-3'	
3'-RACE inner	5'-CGCGGATCCGAATTAATACGACTCACTATAGG-3'	

A forward (F1) and reverse (R1) primer set was designed by selecting highly conserved region of FST mRNA of various avian species, including *Gallus gallus* (GenBank accession number, NM205201), *Falco cherrug* (GenBank accession number, XM005443919), *Taeniopygia guttata* (GenBank accession number, XM002197254.2) and *Zonotrichia albicollis* (GenBank accession number, XM-0055490664). The other primers, excluding RACE primers, F5 and R5, were based on sequence analysis of PCR product from F1-R1 combination. F5 was based on published quail partial FST 5'-terminal cDNA sequence. R5 was based on the sequence obtained from 3'-RACE

ovary total RNA, 3'-RACE adapter and M-MLV reverse transcriptase. Using the RT product, the first 3'-RACE PCR was performed with the 3'-outer RACE primer plus various forward primers (F1, F2, F3 and F4). The F2, F3 and F4 primers (Table 1) were synthesized based on the quail partial FST sequence obtained from the PCR product amplified with F1 and R1 combination. Six PCR products which appeared to contain 3'-terminal regions were excised and purified, followed by another round of PCR reaction with F4 and 3'-inner RACE primer combination, using the purified six fragments as templates. The F4 and 3'-inner RACE primer combination was expected to produce bigger than 267 bp PCR product. The PCR reaction yielded PCR products of same size of 267 bp from all six templates, strongly suggesting the amplification of 3'-terminal region. The PCR product was purified and cloned in pGEM-T Easy vector for sequence analysis. To determine the whole quail FST ORF cDNA sequence, a forward primer (F5 in Table 1) starting from the initiation codon was synthesized based on a published quail partial FST 5'-terminal cDNA sequence (contig-28924 at http://systemsbiology.usm.edu/BirdGenomics/BirdGenomeAnnotation.html). A reverse primer (R5 in Table 1) was also synthesized based on the sequence obtained from 3'-RACE. Using the quail ovary cDNA as a template, a PCR reaction was performed with the F5 and R5 primer combination. The PCR yielded a single amplified product of an expected size (1032 bp), thus the PCR product was purified and cloned in pGEM-T Easy vector, followed by sequence analysis.

To determine the 5' untranslated region of quail FST cDNA, 5'-RACE step was carried out following the protocol described by the manufacture (First Choice® RLM-RACE Kit, Life Technologies, NY, USA). Few 5'-RACE PCR products were obtained, but sequence analysis did not show any matching to FST sequence. The entire ORF sequence of quail FST was obtained, enough information for protein expression, thus we did not pursue the determination of 5' untranslated region of quail FST cDNA.

Nucleotide and amino acid sequence analysis: Translation of the sequenced DNA into amino acid sequence was performed using translation tool available from the ExPASY Bioinformatics Portal. Similarity searches for the sequenced DNA and predicted amino acid sequences were done by Blastn and Blastx on NCBI GenBank database[18]. Pair-wise and multiple sequence alignments of the predicted quail FST protein with the known FST proteins were analyzed using ClustalW2[19].

Cloning of qFST288 into an expression vector and transformation of expression vectors: Previously, pMALc5x vector (New England Biolabs, MA, USA) was successfully used in cloning for expression of bioactive chicken FST315 in an *E. coli* system[20], thus the same system was used in cloning qFST288 in this study. Gibson assembly cloning method[21] was used to insert the DNA fragments of qFST288 into pMALc5x vector separately (New England Biolabs, MA, USA). Gibson assembly primers for FST288 fragments were commercially synthesized: Forward 5'-gggatcgagggaaggGGGAACTGCTG

GCTCCGG-3' and reverse 5'-catggacatatgtgaaatTTAGTTG CAAGATCCAGAGTGCTTTAC-3', in which small capital indicates overlap sequence part and all capital indicates primer sequence part used for Gibson assembly cloning. The overlapping insert was prepared by PCR amplification using the Q5 High-Fidelity PCR kit (New England Biolabs) and qFST full sequence cDNA as a template. The PCR product was separated by agarose gel electrophoresis and fragment was excised and purified before use in DNA assembly reaction with XmnI-linearized pMALc5x vector using Gibson Assembly® Cloning kit (New England Biolabs). Each assembly reaction contained approximately 100 ng of insert and 50 ng of the expression vector and incubated at 50°C for 30 min following the manufacturer's protocol. After the assembly reaction, the reaction mix was transformed into NEB 5-alpha competent *E. coli* strain (New England Biolabs). After an overnight growth at 37°C, the pMAL-c5x plasmid containing qFST288 cDNA (pMALc5x-qFST288) was extracted using a plasmid extraction miniprep kit (Promega) to confirm correct insertion by colony PCR.

Cytoplasmic expression of four FST-type proteins: The K12TB1 or Shuffle *E. coli* strains (New England Biolabs) were transformed with pMALc5x-qFST288 plasmid. After confirmation of correct insertion by colony PCR, 5 mL Luria-Bertani (LB) (1.2% tryptone, 0.6% yeast extract and 0.8% NaCl) medium containing 100 μg mL^{-1} ampicillin and 0.2% glucose were inoculated with the K12TB1 or Shuffle *E. coli* harboring the pMALc5x-qFST288 and grown overnight at 37°C with vigorous shaking. Then, 5 mL of the overnight cultures were transferred into 1 L fresh LB medium containing ampicillin in 2 L flask. For the K12TB1 culture, when the culture reached to an optical density of 0.3-0.4A (600 nm) at 37°C, protein expression was induced at different temperatures by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM under vigorous shaking for different time periods. After induction, *E. coli* pellet was harvested. Each gram of the wet pellet was resuspended in 5 mL of affinity column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) containing the Complete Mini Protease Inhibitor cocktail tablet (Roche, Mannheim, Germany). Two microliters of lysozyme (50 μg mL^{-1}) and 2 μL of DNase I (2500 units mL^{-1}) were added per 1 mL column buffer. The resuspended cell solution was lysed by sonication in short pulses of 15 sec for 10 min in ice water bath. The soluble and insoluble fractions were prepared by centrifugation at 10,000 rpm for 20 min at 4°C. For each sample, the supernatants (soluble fraction) were collected and the same volume of column buffer was used to resuspend the pellets (insoluble fraction). Total, soluble and pellet fractions were analyzed by SDS-PAGE to examine the presence of MBP-fused recombinant qFST288 (MBP-qFST288).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): The SDS-PAGE was performed with gels containing 12.5% polyacrylamide and 0.1% SDS following the procedure of Laemmli[22]. Samples were mixed with 3X loading buffer which were under reducing conditions. Before loading the sample onto the SDS-PAGE gel, samples were boiled at 100°C for 5 min.

Amylase affinity purification of MBP-qFST288: Supernatant cell extracts were diluted with affinity column buffer in a 1:5 ratio and filtered through 0.45 μm filter, then was loaded into an amylose resin column equilibrated with 100 mL of column buffer. After loading, the pass-through was collected and washed with 100 mL of column buffer. Proteins bound to the column were then eluted with elution buffer (column buffer with 10 mM maltose) at a flow rate of 0.5 mL min^{-1}. Five milliliter fractions were collected during elution and the absorbance was monitored at 280 nm. After SDS-PAGE analysis of the presence of recombinant protein in fractions, fractions containing recombinant proteins were pooled.

Heparin affinity purification of MBP-fused FST-type proteins: For further purification of amylose affinity-purified MBP-fused proteins, the pooled elutions were subjected to heparin affinity column (Bio-Rad, CA, USA) previously equilibrated with column buffer. The pass-through was collected at a rate of 1 mL min^{-1}. The column was then washed with 100 mL of column buffer. Proteins bound to the column were then eluted with elution buffer (column buffer with 1M NaCl). After SDS-PAGE analysis of the presence of recombinant protein in fractions, fractions containing recombinant proteins were pooled, followed by dialysis in phosphate buffered saline solution.

Bioactivity test of MBP-qFST288 by pGL3-$(CAGA)_{12}$ Luc-luciferase reporter system: The capacities of MBP-qFST288 to suppress the bioactivity of three FST-binding proteins (MSTN and activin A, all from R&D Systems) were examined by a procedure that was used in examining the bioactivity of MBP-fused chicken FST315[20]. Briefly, cells stably

transformed with pGL3-$(CAGA)_{12}$ luciferase reporter construct[23] were seeded in a 96 well plate for 24 h at 37°C with 5% CO_2. The medium was replaced with 100 µL serum-free DMEM containing 1 nM MSTN or activin A plus various concentrations of MBP-qFST288, then incubated for 24 h. After removing the medium, Bright-Glo luminescence substrate (Promega) was added and luminescence was measured. The % inhibition of MSTN, GDF-11 or activin A activity was calculated by the following formula:

$$\% \text{ Inhibition} = \frac{\left[\begin{array}{c}\text{Luminescence at 1 nM MSTN or activin A-}\\ \text{luminescence at each ligand concentration}\end{array}\right]}{\left[\begin{array}{c}\text{Luminescence at 1 nM MSTN or activin A-}\\ \text{luminescence at 0 nM MSTN or activin}\end{array}\right]} \times 100$$

The MSTN- or activin-inhibitory activity was analyzed by regression analysis using Prism 5 program (Graphpad, C A, USA). To examine the differences in MSTN-or activin A-inhibitory capacity of these proteins, IC_{50} (ligand concentration inhibiting 50% of MSTN or activin A activity) values were estimated using a non-linear regression model defining dose response curve .The equation for the model was as follows:

$$Y = \frac{\text{Bottom} + (\text{Top} - \text{bottom})}{[1 + 10 \wedge (X - \text{Log}IC_{50})]}$$

where, Y is F/R ratio, bottom is the lowest value of F/R ratio, top is the highest value of F/R ratio and X is Log ligand concentration. The IC_{50} values were analyzed by a one-way ANOVA (analysis of variance) using the same program. Statistical significance was accepted at $p<0.05$.

RESULTS

Cloning and molecular characterization of quail FST cDNA: The full-length quail FST (qFST) cDNA was obtained using RACE and it consisted of 1118 bp with an open reading frame of 1032 bp and this cDNA sequence was deposited to GenBank (accession number KJ803018.1). When the qFST cDNA sequence was aligned against chicken sequence (Fig. 1), it shows 98% nucleotide identity to chicken FST cDNA with 21 nucleotides being different out of 1032 nucleotides. The sequence encodes a 343 amino acids protein. Alignment of amino acid sequence of qFST to other avian species, including chicken (*Gallus gallus*), budgerigar (*Melopsittacus undulates)*, saker falcon (*Falco cherrug*), zebra finch (*Taeniopygia guttata*) and white-throated sparrow (*Zonotrichia albicollis*), showed about 98% homology with less than 4 amino acids difference (Fig. 2). The qFST amino acid sequence was identical to chicken FST except the sequence at 28 position (Fig. 2). Since the first 28 amino acid is a signal sequence and being removed after translation to yield FST315, it is indicated that qFST315 is identical to chicken FST315. The qFST315 also revealed high homology to mammalian FST315 such as human and mouse FST315 with more than 95% homology (Fig. 2).

Cytoplasmic expression of MBP-qFST288 and affinity purification: Researches first examined whether MBP-qFST288 could be expressed in a soluble form in K12TB1 strain. The expressed MBP-qFST288 was mostly present in the pellet fraction of cell lysate in SDS-PAGE analysis (Fig. 3), indicating an insoluble expression of the MBP-qFST288 in K12TB1 strain. Researchers, thus, examined the induction in shuffle strain at different temperatures. Both induction at 37°C for 4 h and at 25°C for 8 h resulted in insoluble expression, while induction at 4°C for 8 days resulted in a soluble expression (Fig. 4).

The soluble fraction of cell lysate expressed at 4°C for 8 days were applied to amylose affinity chromatography and fraction were collected and fractions were subjected to SDS-PAGE analysis with samples being in both reduced and non-reduced conditions (Fig. 5a). The purification appeared to be more than 90% and in non-reduced conditions, the MBP-qFST288 remained in the same expected size without aggregation. The fractions containing MBP-qFST288 were combined, then subjected to heparin affinity chromatography. Fractions were also subjected to SDS-PAGE analysis with samples being in both reduced and non-reduced conditions (Fig. 5b). Further purification appeared to occur. The yield of amylose/heparin affinity-purified MBP-qFST288 was around 7 mg L^{-1} culture.

Bioactivity of MBP-qFST288: The abilities of MBP-qFST288 to suppress MSTN and activin A were examined using pGL3-$(CAGA)_{12}$ Luc-luciferase reporter assay (Fig. 6) and their potencies were compared to commercial recombinant human FST produced in eukaryotic cells (rhFST315/CHO, R&D Systems). The IC_{50} value to suppress 1 nM MSTN by MBP-qFST288 was 3.55 nM and this value was significantly higher than that of rhFST315/CHO (0.12 nM). The IC_{50} value to suppress 1 nM activin A by MBP-qFST288 was 1.82 nM and this value was significantly higher than that of rhFST315/CHO (0.18 nM).

```
Q-cDNA  ATGTTAAATCAGAGGATCCACCCGGGCATGCTCGTACTCCTGATGTTTCTCTACCACTTC 60
ch-cDNA ATGTTAAATCAGAGGATCCACCCGGGCATGCTCGTACTCCTGATGTTTCTCTACCACTTC 60
        ************************************************************

Q-cDNA  ATGGAAGATCACACAGCGCTGGCTGGGAACTGCTGGCTCCGGCAGGCACGGAACGGCCGC 120
ch-cDNA ATGGAAGATCACACAGCGCAGGCTGGGAATTGTTGGCTCCGGCAGGCGCGGAACGGCCGC 120
        ****************** ********* ** ************** ************

Q-cDNA  TGCCAGGTCCTCTACAAGACCGACCTCAGCAAAGAGGAGTGCTGCAAGAGCGGCCGCCTG 180
ch-cDNA TGCCAGGTCCTCTACAAGACCGACCTCAGCAAAGAGGAGTGCTGCAAGAGCGGCCGCCTG 180
        ************************************************************

Q-cDNA  ACGACTTCGTGGACGGAGGAGGACGTCAACGACAACACGCTCTTTAAGTGGATGATTTTT 240
ch-cDNA ACGACCTCGTGGACGGAGGAGGACGTCAACGACAACACGCTCTTTAAGTGGATGATTTTT 240
        ***** ******************************************************

Q-cDNA  AATGGGGGAGCCCCTAACTGCATCCCGTGCAAAGAAACATGTGAGAACGTGGACTGTGGA 300
ch-cDNA AATGGGGGAGCCCCTAACTGCATCCCGTGCAAAGAAACATGTGAGAATGTGGACTGTGGA 300
        ********************************************** ************

Q-cDNA  CCCGGGAAGAAATGTAAAATGAACAAGAAGAACAAACCTCGGTGTGTTTGTGCTCCAGAT 360
ch-cDNA CCTGGGAAGAAATGTAAAATGAACAAGAAGAACAAACCTCGGTGTGTTTGTGCTCCGGAT 360
        ** ********************************************************* ***

Q-cDNA  TGCTCTAATATCACCTGGAAGGGCCCCGTGTGTGGCTTAGATGGGAAAACCTACAGGAAC 420
ch-cDNA TGCTCTAATATCACTTGGAAGGGCCCCGTGTGTGGCTTAGATGGGAAAACCTACAGGAAC 420
        ************** ********************************************

Q-cDNA  GAGTGTGCCCTTCTCAAAGCCAGATGTAAAGAACAGCCCGAACTTGAAGTCCAGTATCAG 480
ch-cDNA GAGTGTGCCCTTCTCAAAGCCAGATGTAAAGAACAGCCCGAACTTGAAGTCCAATATCAG 480
        ************************************************** ******

Q-cDNA  GGCAAGTGCAAAAAGACCTGTAGGGATGTTTTATGCCCAGGCAGCTCCACATGTGTGGTG 540
ch-cDNA GGCAAGTGCAAAAAGACCTGTAGGGATGTTTTATGCCCAGGCAGCTCCACGTGTGTGGTG 540
        ************************************************* *********

Q-cDNA  GATCAAACCAATAACGCCTACTGTGTGACATGTAATCGAATTTGCCCTGAGCCTACCTCC 600
ch-cDNA GATCAAACTAATAACGCCTACTGTGTGACATGTAATCGAATTTGCCCTGAGCCTACCTCC 600
        ******** ***************************************************

Q-cDNA  CCTGAACAGTATCTCTGTGGGAATGATGGCATAACTTATGCCAGTGCCTGCCACCTGAGA 660
ch-cDNA CCTGAGCAGTATCTCTGTGGGAATGATGGCATAACTTACGCCAGTGCCTGCCACCTGAGA 660
        ***** ********************************* ********************

Q-cDNA  AAAGCGACCTGCCTGCTGGGCAGATCCATTGGATTAGCCTACGAGGGAAAATGCATCAAA 720
ch-cDNA AAAGCGACCTGCCTGCTGGGCAGATCCATTGGATTAGCCTACGAGGGAAAATGCATCAAA 720
        ************************************************************

Q-cDNA  GCGAAGTCCTGTGAAGATATTCAATGCAGTGCTGGGAAGAAATGCTTGTGGGATTTTAAG 780
ch-cDNA GCGAAGTCCTGTGAAGATATTCAGTGCAGTGCTGGGAAGAAATGCTTGTGGGATTTTAAG 780
        ********************** ***********************************

Q-cDNA  GTTGGCAGAGGTCGATGTGCCCTCTGTGATGAGCTCTGCCCTGAAAGCAAGTCAGACGAG 840
ch-cDNA GTTGGCAGAGGTCGATGTGCCCTCTGTGATGAGCTCTGCCCTGAAAGCAAGTCAGATGAG 840
        ********************************************************* ***

Q-cDNA  GCAGTCTGTGCCAGTGATAACACGACTTACCCGAGCGAGTGTGCCATGAAGGAGGCAGCC 900
ch-cDNA GCAGTCTGTGCCAGTGATAACACAACTTATCCGAGCGAGTGTGCCATGAAAGAGGCAGCC 900
        ********************** ***** ******************** *********

Q-cDNA  TGCTCCATGGGCGTGCTTCTAGAAGTAAAGCACTCTGGATCTTGCAACTCCATTAATGAA 960
ch-cDNA TGTTCCATGGGCGTGCTTCTAGAAGTAAAGCACTCTGGATCTTGCAACTCCATTAACGAA 960
        ** ************************************************** ***

Q-cDNA  GACCCAGAGGAAGAAGAGGAAGATGAAGACCAGGACTACAGCTTTCCTATATCTTCCATT 1020
ch-cDNA GACCCAGAGGAAGAAGAGGAAGATGAAGACCAGGACTACAGCTTTCCTATATCTTCCATT 1020
        ************************************************************

Q-cDNA  CTAGAGTGGTAA 1032
ch-cDNA CTAGAGTGGTAA 1032
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Fig. 1: Quail FST full-length cDNA (Q-cDNA) was aligned against chicken FST cDNA (ch-cDNA) using CLUSTALW

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Coturnix      MLNQRIHPG-MLVLLMFLYHFMEDHTALAGNCWLRQARNGRCQVLYKTDLSKEECCKSGR 59
Gallus        MLNQRIHPG-MLVLLMFLYHFMEDHTAQAGNCWLRQARNGRCQVLYKTDLSKEECCKSGR 59
Taeniopygia   MLNQRIHPG-MLLILMFLCHFMEDHTVQAGNCWLRQARNGRCQVLYKTDLSKEECCKSGR 59
Zonotrichia   MLNQRIHPG-MLLILMFLCHFMEDHTVQAGNCWLRQARNGRCQVLYKTDLSKEECCKSGR 59
Melopsittacus MLNQRIHPG-MLLLLMFLCHFMEDHTVQAGNCWLRQARNGRCQVLYKTDLSKEECCKTGR 59
Falco         MLNQRIHPG-MLLLLMFLCHFMEDHTVQAGNCWLRQARNGRCQVLYKTDLSKEECCKTGR 59
Homo          MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTELSKEECCSTGR 60
Mus           MVCARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTELSKEECCSTGR 60
              *: * :** : ::*::* :****::. *********:**********:*******.:**

Coturnix      LTTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCKMNKKNKPRCVCAP 119
Gallus        LTTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCKMNKKNKPRCVCAP 119
Taeniopygia   LTTSWTAEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCKMNKKNKPRCVCAP 119
Zonotrichia   LTTSWTAEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCKMNKKNKPRCVCAP 119
Melopsittacus LTTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCKMNKKNKPRCVCAP 119
Falco         LTTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCKMNKKNKPRCVCAP 119
Homo          LSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAP 120
Mus           LSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAP 120
              *:**** ************************************:*************

Coturnix      DCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGKCKKTCRDVLCPGSSTCV 179
Gallus        DCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGKCKKTCRDVLCPGSSTCV 179
Taeniopygia   DCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGKCKKTCRDVLCPGSSTCV 179
Zonotrichia   DCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGKCKKTCRDVLCPGSSTCV 179
Melopsittacus DCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGKCKKTCRDVLCPGSSTCV 179
Falco         DCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGKCKKTCRDVLCPGSSTCV 179
Homo          DCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCV 180
Mus           DCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGKCKKTCRDVFCPGSSTCV 180
              ****************************************:********:********

Coturnix      VDQTNNAYCVTCNRICPEPTSPEQYLCGNDGITYASACHLRKATCLLGRSIGLAYEGKCI 239
Gallus        VDQTNNAYCVTCNRICPEPTSPEQYLCGNDGITYASACHLRKATCLLGRSIGLAYEGKCI 239
Taeniopygia   VDQTNNAYCVTCNRICPEPTSAEQYLCGNDGITYASACHLRKATCLLGRSIGLAYEGKCV 239
Zonotrichia   VDQTNNAYCVTCNRICPEPTSPEQYLCGNDGITYASACHLRKATCLLGRSIGLAYEGKCV 239
Melopsittacus VDQTNNAYCVTCNRICPEPTSPEQYLCGNDGITYASACHLRKATCLLGRSIGLAYEGKCI 239
Falco         VDQTNNAYCVMCNRICPEPTSPEQYLCGNDGITYASACHLRKATCLLGRSIGLAYEGKCI 239
Homo          VDQTNNAYCVTCNRICPEPASSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCI 240
Mus           VDQTNNAYCVTCNRICPEPSSSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCI 240
              ********** ********:*.*********:**:************************:

Coturnix      KAKSCEDIQCSAGKKCLWDFKVGRGRCALCDELCPESKSDEAVCASDNTTYPSECAMKEA 299
Gallus        KAKSCEDIQCSAGKKCLWDFKVGRGRCALCDELCPESKSDEAVCASDNTTYPSECAMKEA 299
Taeniopygia   KAKSCEDIQCSAGKKCLWDFKVGRGRCALCDELCPESKSEEAVCASDNTTYPSECAMKEA 299
Zonotrichia   KAKSCEDIQCSAGKKCLWDFKVGRGRCALCDEMCPESKSEEAVCASDNTTYPSECAMKEA 299
Melopsittacus KAKSCEDIQCSAGKKCLWDFKVGRGRCALCDELCPESKSDEAVCASDNTTYPSECAMKEA 299
Falco         KAKSCEDIQCSAGKKCLWDFKVGRGRCALCDELCPESKSDEAVCASDNTTYPSECAMKEA 299
Homo          KAKSCEDIQCTGGKKCLWDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEA 300
Mus           T-KSCEDIQCGGGKKCLWDSKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEA 299
              . ******** .******* *******:****:**:***:*.******:**.********

Coturnix      ACSMGVLLEVKHSGSCNSINEDPEEEEEDEDQDYSFPISSILEW 343
Gallus        ACSMGVLLEVKHSGSCNSINEDPEEEEEDEDQDYSFPISSILEW 343
Taeniopygia   ACSMGVLLEVKHSGSCNSINEDPEDEEEDEDQDYSFPISSILEW 343
Zonotrichia   ACSMGVLLEVKHSGSCNSINEDPEDEEEDEDQDYSFPISSILEW 343
Melopsittacus ACSMGVLLEVKHSGSCNSINEDPEDEDEDEDQDYSFPISSILEW 343
Falco         ACSMGVLLEVKHSGSCNSINEDPEDEEEDEDQDYSFPISSILEW 343
Homo          ACSSGVLLEVKHSGSCNSISEDTEEEEEDEDQDYSFPISSILEW 344
Mus           ACSSGVLLEVKHSGSCNSISEETEEEEEEEDQDYSFPISSILEW 343
              *** ***************.*:.*:*:*:*:***************
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Fig. 2: Alignment of quail FST amino acid sequence to various animal species. Amino acid sequence of quail FST (*Coturnix japonica*, accession number KJ803018.1) was aligned to those of chicken (*Gallus gallus*, accession number NM_205200.1), budgerigar (*Melopsittacus undulates*, accession number XM_005151955.1), saker falcon (*Falco cherrug*, accession number XM_005443919.1), zebra finch (*Taeniopygia guttata*, accession number XM_002197254.2), white-throated sparrow (*Zonotrichia albicollis*, accession number, XM_005490664.1), human (*Homo sapiens*, accession number BC004107.2), house mouse (*Mus musculus*, accession number, Z29532.1)

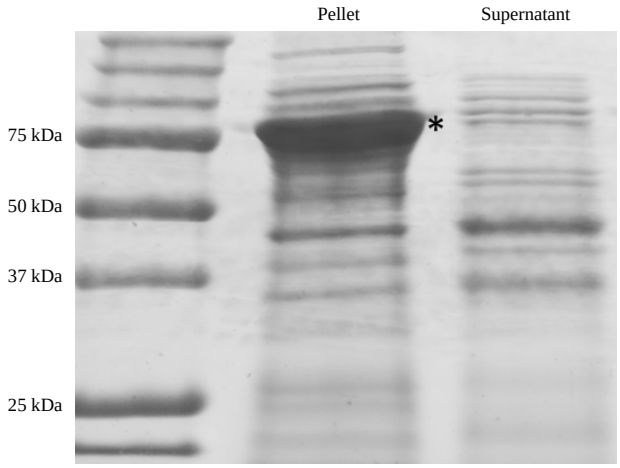

Fig. 3: SDS-PAGE analysis of MBP-qFST288 expressed in K12TB1 *E. coli* strain at 37°C. Induced cell lysate was centrifuged at 10,000 rpm for 5 min to separate insoluble (pellet) and soluble (supernatant) fractions, then the each fraction was subjected to 12.5% SDS-PAGE and stained with coomassie blue to examine the expression of MBP-qFST288. Asterisk (*) indicates the MBP-qFST288

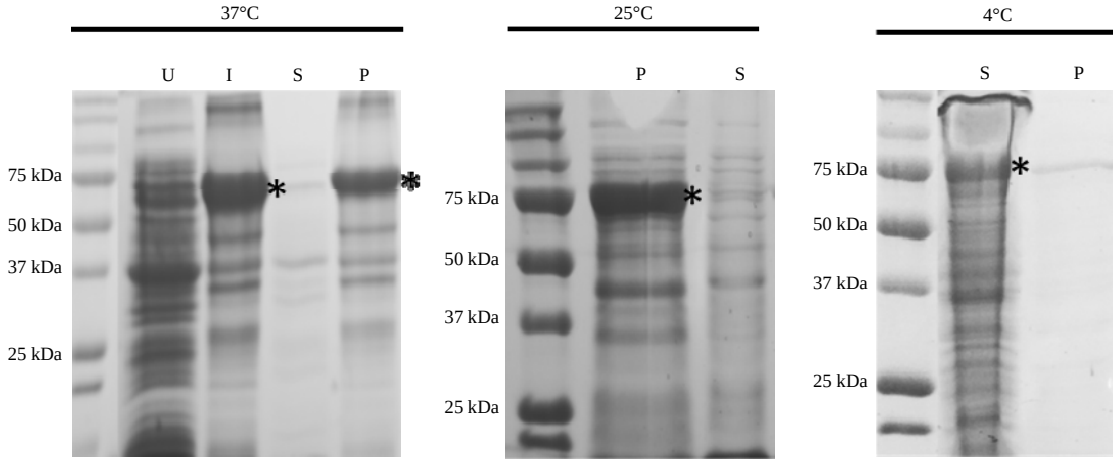

Fig. 4: SDS-PAGE analysis of MBP-qFST288 expressed in shuffle *E. coli* strain at 37, 25 or 4°C. Induced cell lysate was centrifuged at 10,000 rpm for 5 min to separate insoluble (pellet) and soluble (supernatant) fractions, then the each fraction was subjected to 12.5% SDS-PAGE and stained with Coomassie blue to examine the expression of MBP-qFST288. Asterisk (*) indicates the MBP-qFST288. U: Un-induced fraction, I: Induced fraction, S: Supernatant (soluble) fraction, P: Pellet (insoluble) fraction

DISCUSSION

The quail full-length follistatin (FST) cDNA sequence has not been available, thus in the current study, the full-length quail FST cDNA consisting of 1118 bp was cloned with an open reading frame of 1032 bp and sequenced the cDNA (GenBank accession number KJ803018.1). Amino acid sequence of quail FST shows 98% homology with other avian species, including chicken, budgerigar, saker falcon, zebra finch and white-throated sparrow, demonstrating a tight evolutionary conservation of avian FST. It was also revealed that the amino acid sequence of quail FST315 is the same as the chicken FST315.

The FST is a multi-domain glycoprotein consisting of 5 domains, including N-terminal domain followed by three FST domains (FSD1-3) and C-terminal domain [24]. Three isoforms of FST, including FST315, FST303 and FST288, have been identified *in vivo*[25]. The FST315 encompasses all domains and FST288 is lacking the C-terminal domain and FST303 arises from proteolytic cleavage of the FST315 C-terminal tail[26].

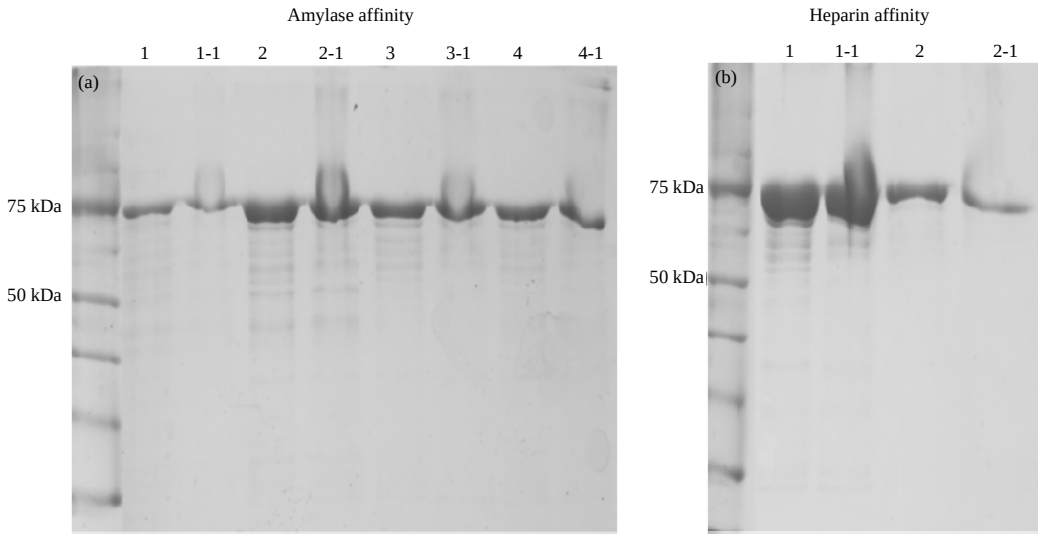

Fig. 5: SDS-PAGE analysis of MBP-qFST288 after amylose and heparin affinity purifications. MBP-qFST288 was expressed in shuffle *E. coli* strain at 4°C for 8 days. Soluble fraction of cell lysate was applied to (a) Amylose affinity chromatography and combined elutions were subjected to (b) Heparin affinity chromatography. Elutions were examined by SDS-PAGE for the presence of qFST288 (B). Lanes 1, 2, 3 and 4 are elution fractions in reduced loading buffer and lanes 1-1, 2-1, 3-1 and 4-1 are elution fractions in non-reduced loading buffer

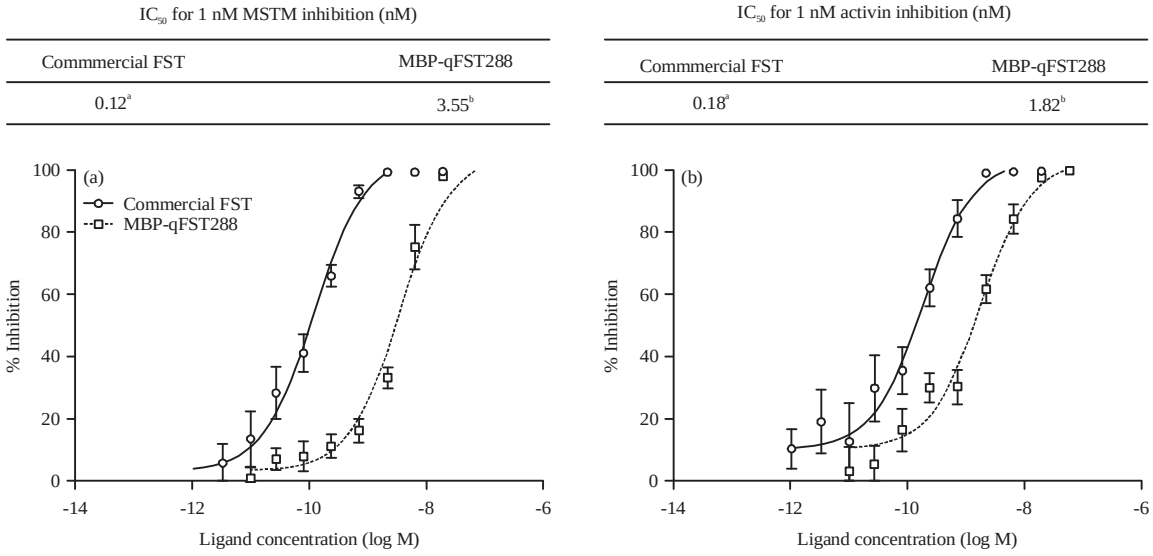

Fig. 6: Inhibition of (a) MSTN or (b) Activin A activities by MBP-qFST288. The HEK293 cells stably expressing $(CAGA)_{12}$-luciferase gene construct were seeded on a 96-well culture and grown for 24 h in DMEM with 10% fetal calf serum, antibiotic and antimycotic. Medium was removed and MSTN (1 nM) or activin A (1 nM) plus various concentrations (180-0 nM) of MBP-qFST288 in DMEM were added to each well, followed by incubation for 24 h. Medium was removed and luminescence substrate was added, followed by luminescence measurement. The error bars represent ± SEM (n = 6)

These isoforms have shown locational compartmentalization and different biological roles *in vivo*[25, 27]. The longest FST 315 isoform is found in the circulation, whereas, the shortest FST288 isoform is typically found in or on cells and tissues and the intermediate FST303 isoform is found in gonads[27]. The length of C-terminal domain at FST determines the activity of the heparin binding sequence within FST domain 1 such that FST288 binds with high affinity, FST303 with medium affinity and FST315 with low affinity to cell surface heparin-sulfated proteoglycans[26,28]. This leads to a suggestion that the FST288

isoform would be superior to the other isoforms in regulating autocrine or paracrine-acting activin or other TGF-beta superfamily proteins within tissues or organs[25] and it has been shown that FST288 was eight to ten times more potent than FST315 in suppressing FSH secretion in a rat anterior pituitary cell culture[28].

Recently, bioactive FST315 was produced in a soluble form in *E. coli* using maltose-binding protein as a fusion partner[20], thus in this study, researchers examined whether FST288 can be produced using the same system. The results show that bioactive MBP-FST288 can also be produced in an *E. coli* system. The yield of amylose-heparin affinity purified MBP-FST288 (7 mg L^{-1} culture) was comparable to that of FST315 (6 mg L^{-1} culture)[20]. Like the MBP-FST315, the potency of MBP-FST288 to suppress the activities of MSTN or activin A was lower than human FST315 of eukaryotic cell origin. Cleavage of MBP from MBP-FST315 had no significant effect on its bioactivity to suppress MSTN or activin A[20]. It is, thus likely that the presence of MBP as a fusion partner is not a factor for the lower potency of MBP-FST288 as compared with FST315 of eukaryotic cell origin. It is possible that the lack of glycosylation and/or the amino acid sequence difference between the quail and human FST contributed to the affinity difference. The IC_{50} value of MBP-FST288 (3.55 nM) to suppress 1 nM MSTN appeared higher to be lower than that of MBP-FST288 (8.85 nM)[20], indicating that the potencies of MBP-FST288 to suppress MSTN is higher than that of MBP-FST315. The IC_{50} value of MBP-FST288 (1.82 nM) to suppress 1 nM activin A appeared to be close to that of MBP-FST315 (1.86 nM)[20], indicating that the potencies of MBP-FST315 and MBP-FST288 to suppress activin A are similar. In disagreement of current result, other studies have shown that mammalian FST288 of eukaryotic cell origin had a higher affinity or inhibitory potency for activin A than mammalian FST315 of eukaryotic origin[28,29].

CONCLUSION

Researchers cloned the full-length quail FST cDNA and found out that the amino acid sequence of quail FST315 is the same as the chicken FST315. Furthermore, researchers produced bioactive quail FST288 in an *E. coli* system and the easy availability of FST288 can contribute to examining the future potentials of FST288 as an agent to improve skeletal muscle growth of poultry.

SIGNIFICANCE STATEMENT

This study revealed the sequence for quail follistatin and demonstrated its functional biological activity that can be beneficial for further studies aimed at reducing myostatin expression in quail.

This study will provide the demonstrated ability for researchers to synthesize a compound that may be used to enhance the size of quail.

ACKNOWLEDGMENT

Supported partially by a grant from Ministry of Higher Education and Scientific Research Iraq (YM) and USDA-NIFA grant 2010-34135-21229 (YK). Support also partially provided by USDA Regional Project NC-01084 (PEM and YK).

Supplement 1: CLUSTAL O (1.2.1) multiple sequence alignment

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Chicken       ATGTTAAATCAGAGGATCCACCCGGGCATGCTCGTACTCCTGATGTTTCTCTACCACTTC  60
Falco         ATGTTAAATCAGAGAATCCACCCGGGCATGCTCTTACTCCTGATGTTTCTGTGCCACTTC  60
Zonotrichia   ATGTTAAATCAGAGAATCCACCCGGGCATGCTCTTAATCCTGATGTTTCTGTGCCACTTC  60
Taeniopygia   ATGTTAAATCAGAGAATCCACCCGGGCATGCTCTTAATCCTGATGTTTCTGTGCCACTTC  60
              ************** ********************** ** ************ * *******

Chicken       ATGGAAGATCACACAGCGCAGGCTGGGAATTGTTGGCTCCGGCAGGCGCGGAACGGCCGC  120
Falco         ATGGAAGATCACACAGTGCAGGCTGGGAACTGCTGGCTCCGGCAGGCGCGGAACGGCCGC  120
Zonotrichia   ATGGAAGATCACACAGTGCAGGCTGGGAACTGCTGGCTCCGGCAGGCGCGGAACGGGCGC  120
Taeniopygia   ATGGAAGATCACACAGTGCAGGCTGGGAACTGCTGGCTCCGCCAGGCGCGGAACGGGCGC  120
              ***************** ************ ** ******** ************** ***

Chicken       TGCCAGGTCCTCTACAAGACCGACCTCAGCAAAGAGGAGTGCTGCAAGAGCGGCCGCCTG  180
Falco         TGCCAGGTCCTCTACAAAACCGACCTCAGCAAGGAGGAGTGCTGCAAGACCGGCCGCCTG  180
Zonotrichia   TGCCAGGTCCTCTACAAAACCGACCTCAGCAAGGAGGAGTGCTGCAAGAGCGGCCGCCTG  180
Taeniopygia   TGCCAGGTCCTCTACAAAACCGACCTCAGCAAGGAGGAGTGCTGCAAGAGCGGCCGCCTG  180
              ****************** ************** ****************** **********

Chicken       ACGACCTCGTGGACGGAGGAGGACGTCAACGACAACACGCTCTTTAAGTGGATGATTTTT  240
Falco         ACAACTTCGTGGACGGAAGAGGACGTCAACGACAACACTCTTTTTAAGTGGATGATTTTT  240
Zonotrichia   ACCACGTCCTGGACGGCGGAGGACGTCAATGACAACACGCTTTTCAAGTGGATGATTTTT  240
Taeniopygia   ACCACGTCGTGGACGGCGGAGGACGTCAACGACAATACGCTTTTTAAGTGGATGATTTTT  240
              ** ** ** ******* *********** ***** ** ** ** ***************
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Supplement 1: Continue

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Chicken       AATGGGGGAGCCCCTAACTGCATCCCGTGCAAAGAAACATGTGAGAATGTGGACTGTGGA 300
Falco         AATGGGGGAGCCCCAAACTGCATCCCGTGCAAAGAAACGTGCGAGAACGTGGACTGTGGA 300
Zonotrichia   AATGGGGGAGCCCCAAACTGCATCCCGTGCAAAGAAACATGCGAGAATGTGGACTGTGGA 300
Taeniopygia   AATGGGGGAGCCCCAAACTGCATCCCATGCAAAGAAACATGCGAGAATGTGGACTGTGGA 300
              ********************** ********** ********** ** ***** ***********

Chicken       CCTGGGAAGAAATGTAAAATGAACAAGAAGAACAAACCTCGGTGTGTTTGTGCTCCGGAT 360
Falco         CCCGGGAAGAAATGTAAAATGAACAAGAAGAACAAACCTCGGTGTGTTTGTGCTCCGGAT 360
Zonotrichia   CCCGGGAAGAAATGTAAAATGAACAAGAAGAACAAACCTCGGTGTGTTTGTGCTCCGGAT 360
Taeniopygia   CCCGGGAAGAAATGTAAAATGAACAAGAAGAACAAACCTCGGTGTGTTTGTGCTCCGGAT 360
              ** ************************************************************

Chicken       TGCTCTAATATCACTTGGAAGGGCCCCGTGTGTGGCTTAGATGGGAAAACCTACAGGAAC 420
Falco         TGCTCTAATATCACTTGGAAGGGTCCCGTGTGTGGCTTAGATGGGAAAACCTACAGGAAC 420
Zonotrichia   TGCTCTAATATCACTTGGAAGGGCCCCGTGTGTGGCTTAGATGGGAAAACCTACAGGAAC 420
Taeniopygia   TGCTCTAATATCACTTGGAAGGGCCCCGTGTGTGGCTTAGATGGGAAAACCTACAGGAAC 420
              ********************** ************************************

Chicken       GAGTGTGCCCTTCTCAAAGCCAGATGTAAAGAACAGCCCGAACTTGAAGTCCAATATCAG 480
Falco         GAGTGTGCCCTTCTCAAAGCCAGATGTAAAGAACAGCCCGAACTTGAAGTCCAGTATCAG 480
Zonotrichia   GAGTGCGCCCTTCTCAAAGCCAGATGTAAAGAACAGCCCGAACTTGAAGTCCAGTATCAG 480
Taeniopygia   GAGTGCGCCCTTCTCAAAGCCAGATGTAAAGAACAGCCTGAACTTGAAGTCCAGTATCAG 480
              ***** ********************************** ************** ******

Chicken       GGCAAGTGCAAAAAGACCTGTAGGGATGTTTTATGCCCAGGCAGCTCCACGTGTGTGGTG 540
Falco         GGCAAATGCAAAAAGACCTGTAGGGATGTTTTATGCCCAGGCAGCTCCACATGTGTGGTT 540
Zonotrichia   GGCAAATGCAAAAAGACCTGCAGGGATGTCTTATGCCCAGGCAGCTCCACGTGTGTGGTT 540
Taeniopygia   GGCAAATGCAAAAAAACCTGCAGAGATGTCTTATGCCCAGGCAGCTCCACATGTGTGGTT 540
              ***** ******** ***** ** ***** ******************** ********

Chicken       GATCAAACTAATAACGCCTACTGTGTGACATGTAATCGAATTTGCCCTGAGCCTACCTCC
              600
Falco         GACCAAACTAATAATGCCTACTGTGTGATGTGTAATCGAATTTGCCCAGAGCCTACCTCC 600
Zonotrichia   GACCAAACCAACAATGCCTACTGCGTGACATGTAACCGCATTTGTCCAGAGCCTACCTCC 600
Taeniopygia   GACCAAACCAACAATGCATACTGCGTGACATGTAACCGAATTTGTCCAGAGCCTACCTCC 600
              ** ***** ** ** ** ***** **** ***** ** ***** ** ***********

Chicken       CCTGAGCAGTATCTCTGTGGGAATGATGGCATAACTTACGCCAGTGCCTGCCACCTGAGA 660
Falco         CCTGAACAGTATCTCTGCGGGAATGACGGCATAACTTACGCCAGTGCTTGCCACCTGAGG 660
Zonotrichia   CCTGAACAGTATCTCTGCGGGAATGACGGCATAACTTACGCCAGTGCCTGCCACCTGAGG 660
Taeniopygia   GCTGAACAGTATCTCTGTGGGAATGACGGCATAACTTACGCCAGTGCCTGCCACCTGAGG 660
              **** *********** ******** ******************** ***********

Chicken       AAAGCGACCTGCCTGCTGGGCAGATCCATTGGATTAGCCTACGAGGGAAAATGCATCAAA 720
Falco         AAAGCTACCTGCCTACTAGGCAGATCCATTGGATTAGCCTACGAAGGAAAATGCATCAAA 720
Zonotrichia   AAAGCTACCTGCCTCCTGGGAAGATCCATTGGATTAGCCTACGAAGGAAAATGCGTCAAA 720
Taeniopygia   AAAGCTACCTGCCTCCTGGGAAGATCCATTGGATTAGCCTATGAAGGAAAATGTGTCAAA 720
              ***** ******** ** ** ******************** ** ******** *****

Chicken       GCGAAGTCCTGTGAAGATATTCAGTGCAGTGCTGGGAAGAAATGCTTGTGGGATTTTAAG 780
Falco         GCCAAATCCTGTGAAGACATTCAATGCAGTGCTGGGAAGAAATGCTTGTGGGATTTTAAG 780
Zonotrichia   GCCAAATCCTGTGAAGACATTCAATGCAGTGCTGGGAAGAAATGCTTGTGGGATTTTAAG 780
Taeniopygia   GCCAAATCCTGTGAAGACATTCAATGCAGTGCTGGGAAGAAATGCTTGTGGGATTTTAAG 780
              ** ** *********** ***** ************************************

Chicken       GTTGGCAGAGGTCGATGTGCCCTCTGTGATGAGCTCTGCCCTGAAAGCAAGTCAGATGAG 840
Falco         GTTGGCAGAGGTCGGTGCGCCCTCTGCGATGAGCTATGCCCTGAAAGCAAGTCAGACGAG 840
Zonotrichia   GTTGGCAGAGGTCGGTGTGCTCTCTGTGATGAGATGTGCCCTGAAAGCAAGTCAGAAGAA 840
Taeniopygia   GTTGGCAGAGGTCGGTGTGCCCTCTGCGATGAGCTATGCCCTGAAAGCAAGTCAGAAGAA 840
              ************** ** ** ***** ****** * ******************** **
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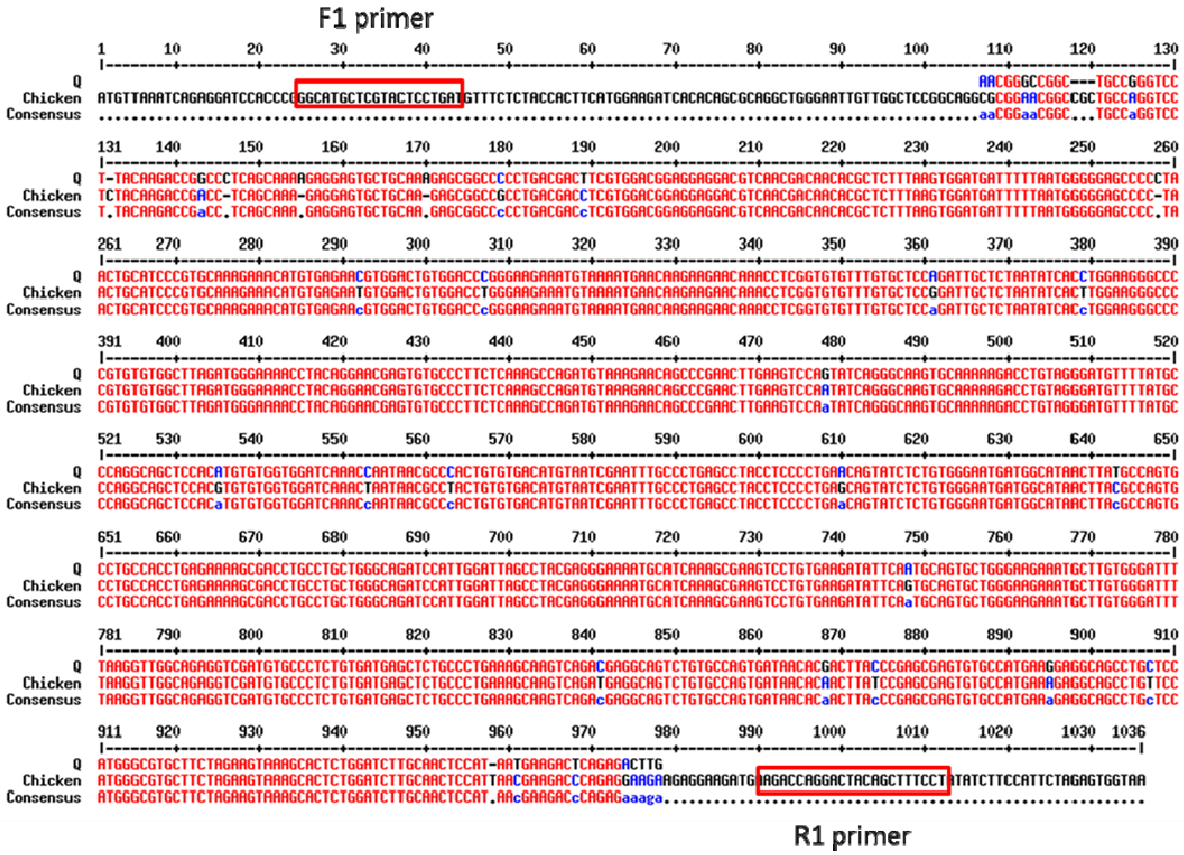
Supplement 1: Continue

Chicken	GCAGTCTGTGCCAGTGATAACACAACTTATCCGAGCGAGTGTGCCATGAAAGAGGCAGCC	900
Falco	GCAGTCTGTGCCAGCGATAACACAACTTACCCAAGCGAGTGTGCCATGAAAGAGGCAGCC	900
Zonotrichia	GCAGTGTGTGCCAGCGATAACACAACTTACCCAAGCGAGTGTGCCATGAAAGAGGCAGCT	900
Taeniopygia	GCAGTGTGTGCCAGCGATAACACAACTTACCCAAGCGAGTGTGCCATGAAAGAGGCAGCT	900
	***** ******** ************** ** *************************	
Chicken	TGTTCCATGGGCGTGCTTCTAGAAGTAAAGCACTCTGGATCTTGCAACTCCATTAACGAA	960
Falco	TGCTCCATGGGTGTGCTTCTAGAAGTAAAGCACTCTGGATCTTGCAACTCCATTAATGAA	960
Zonotrichia	TGCTCCATGGGTGTGCTTCTAGAAGTTAAGCACTCTGGATCTTGCAACTCAATTAATGAA	960
Taeniopygia	TGCTCCATGGGTGTGCTTCTAGAAGTTAAGCACTCTGGATCTTGCAACTCAATTAATGAA	960
	** ******** ************** ********************** ***** ***	
Chicken	GACCCAGAGGAAGAAGAGGAAGATGAAGACCAGGACTACAGCTTTCCTATATCTTCCATT	1020
Falco	GACCCAGAGGATGAAGAGGAAGATGAAGACCAGGACTACAGCTTTCCTATATCTTCCATT	1020
Zonotrichia	GACCCAGAGGATGAAGAGGAAGATGAAGACCAGGACTACAGCTTTCCTATATCTTCCATT	1020
Taeniopygia	GACCCAGAAGATGAAGAGGAAGATGAAGACCAGGACTACAGCTTTCCTATATCTTCCATT	1020
	******** ** **	
Chicken	CTAGAGTGGTAA	1032
Falco	CTAGAGTGGTAA	1032
Zonotrichia	CTAGAGTGGTAA	1032
Taeniopygia	CTAGAGTGGTAA	1032
	************	

The highlighted sequences indicate forward (F1) and reverse (R1) primers used in PCR amplification of quail FST cDNA fragment for the determination of partial quail FST cDNA sequence

Supplement 2:



Alignment of partial quail FST cDNA sequence obtained by sequence analysis of PCR product with F1-R1 primer sets. After sequence analysis, the obtained sequence was aligned against chicken sequence by MultAline

REFERENCES

1. Webster, A.J.F., 1977. Selection for leanness and the energetic efficiency of growth in meat animals. Proc. Nutr. Soc., 36: 53-59.
2. Lee, S.J., 2004. Regulation of muscle mass by myostatin. Annu. Rev. Cell Dev. Biol., 20: 61-86.
3. McPherron, A.C., A.M. Lawler and S.J. Lee, 1997. Regulation of skeletal muscle mass in mice by a new TGF-p superfamily member. Nature, 387: 83-90.
4. Bogdanovich, S., T.O. Krag, E.R. Barton, L.D. Morris, L.A. Whittemore, R.S. Ahima and T.S. Khurana, 2002. Functional improvement of dystrophic muscle by myostatin blockade. Nature, 420: 418-421.
5. Grobet, L., D. Pirottin, F. Farnir, D. Poncelet and L.J. Royo *et al*., 2003. Modulating skeletal muscle mass by postnatal, muscle specific inactivation of the myostatin gene. Genesis, 35: 227-238.
6. Lee, S.J. and A.C. McPherron, 2001. Regulation of myostatin activity and muscle growth. Proc. Natl. Acad. Sci. USA., 98: 9306-9311.
7. Morine, K.J., L.T. Bish, K. Pendrak, M.M. Sleeper, E.R. Barton and H.L Sweeney, 2010. Systemic myostatin inhibition via liver-targeted gene transfer in normal and dystrophic mice. Plos One, Vol. 5. 10.1371/journal.pone.0009176.
8. Tang, L., Z. Yan, Y. Wan, W. Han and Y. Zhang, 2007. Myostatin DNA vaccine increases skeletal muscle mass and endurance in mice. Muscle Nerve, 36: 342-348.
9. Kim, Y.S., N.K. Bobbili, K.S. Paek and H.J. Jin, 2006. Production of a monoclonal anti-myostatin antibody and the effects of *in ovo* administration of the antibody on posthatch broiler growth and muscle mass. Poult. Sci., 85: 1062-1071.
10. Hill, J.J., M.V. Davies, A.A. Pearson, J.H. Wang, R.M. Hewick, N.M. Wolfman and Y. Qiu, 2002. The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. J. Biol. Chem., 277: 40735-40741.
11. Hill, J.J., Y. Qiu, R.M. Hewick and N.M. Wolfman, 2003. Regulation of myostatin *in vivo* by growth and differentiation factor-associated serum protein-1: A novel protein with protease inhibitor and follistatin domains. Mol. Endocrinol., 17: 1144-1154.
12. Gilson, H., O. Schakman, S. Kalista, P. Lause, K. Tsuchida and J.P. Thissen, 2009. Follistatin induces muscle hypertrophy through satellite cell proliferation and inhibition of both myostatin and activin. Am. J. Physiol. Endocrinol. Metabolism, 297: E157-E164.
13. Haidet, A.M., L. Rizo, C. Handy, P. Umapathi and A. Eagle *et al*., 2008. Long-term enhancement of skeletal muscle mass and strength by single gene administration of myostatin inhibitors. Proc. Nat. Acad. Sci., 105: 4318-4322.
14. Kota, J., C.R. Handy, A.M. Haidet, C.L. Montgomery and A. Eagle *et al*., 2009. Follistatin gene delivery enhances muscle growth and strength in nonhuman primates. Sci. Translational Med., Vol. 1. 10.1126/scitranslmed.3000112.
15. Nakatani, M., Y. Takehara, H. Sugino, M. Matsumoto and O. Hashimoto *et al*., 2008. Transgenic expression of a myostatin inhibitor derived from follistatin increases skeletal muscle mass and ameliorates dystrophic pathology in mdx mice. FASEB J., 22: 477-487.
16. Medeiros, E.F., M.P. Phelps, F.D. Fuentes and T.M. Bradley, 2009. Overexpression of follistatin in trout stimulates increased muscling. Am. J. Physiol.-Regul. Integr. Comparat. Physiol., 297: R235-R242.
17. Connolly, D.J., K. Patel, E.A.P. Seleiro, D.G. Wilkinson and J. Cooke, 1995. Cloning, sequencing and expressional analysis of the chick homologue of follistatin Genesis, 17: 65-77.
18. Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman, 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucl. Acids Res., 25: 3389-3402.
19. Larkin, M.A., G. Blackshields, N.P. Brown, R. Chenna and P.A. McGettigan *et al*., 2007. Clustal W and clustal X version 2.0. Bioinformatics, 23: 2947-2948.
20. Lee, S.B., R. Choi, S.K. Park and Y.S. Kim, 2014. Production of bioactive chicken follistatin315 in *Escherichia coli*. Applied Microbiol. Biotechnol., 98: 10041-10051.
21. Gibson, D.G., L. Young, R.Y. Chuang, J.C. Venter, C.A. Hutchison III and H.O. Smith, 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods, 6: 343-345.
22. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
23. Cash, J.N., E.B. Angerman, C. Kattamuri, K. Nolan and H. Zhao *et al*., 2012. Structure of Myostatin• Follistatin-like 3 N-terminal domains of follistatin-type molecules exhibit alternate modes of binding. J. Biol. Chem., 287: 1043-1053.
24. Shimasaki, S., M. Koga, F. Esch, K. Cooksey and M. Mercado *et al*., 1988. Primary structure of the human follistatin precursor and its genomic organization. Proc. Nat. Acad. Sci., 85: 4218-4222.
25. Schneyer, A.L., Q. Wang, Y. Sidis and P.M. Sluss, 2004. Differential distribution of follistatin isoforms: Application of a new FS315-specific immunoassay. J. Clin. Endocrinol. Metab., 89: 5068-5075.

26. Sugino, K., N. Kurosawa, T. Nakamura, K. Takio and S. Shimasaki *et al*., 1993. Molecular heterogeneity of follistatin, an activin-binding protein. Higher affinity of the carboxyl-terminal truncated forms for heparan sulfate proteoglycans on the ovarian granulosa cell. J. Biol. Chem., 268: 15579-15587.
27. Kimura, F., Y. Sidis, L. Bonomi, Y. Xia and A. Schneyer, 2009. The follistatin-288 isoform alone is sufficient for survival but not for normal fertility in mice. Endocrinology, 15: 1310-1319.
28. Inouye, S., Y. Guo, L. Depaolo, M. Shimonaka, N. Ling and S. Shimasaki, 1991. Recombinant expression of human follistatin with 315 and 288 amino acids: Chemical and biological comparison with native porcine follistatin. Endocrinology, 129: 815-822.
29. Hashimoto, O., N. Kawasaki, K. Tsuchida, S. Shimasaki, T. Hayakawa and H. Sugino, 2000. Difference between follistatin isoforms in the inhibition of activin signalling: Activin neutralizing activity of follistatin isoforms is dependent on their affinity for activin. Cell. Signall., 12: 565-571.