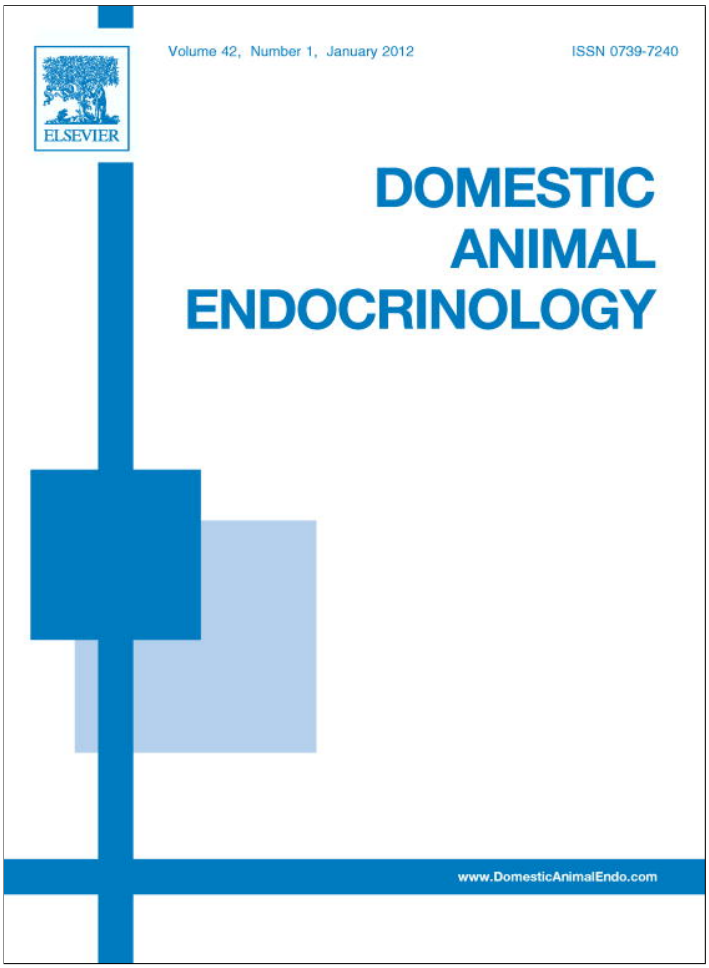

Available online at www.sciencedirect.com

SciVerse ScienceDirect

Domestic Animal Endocrinology 42 (2012) 1–10

DOMESTIC ANIMAL ENDOCRINOLOGY

www.domesticanimalendo.com

Cloning and characterization of chicken fat mass and obesity associated (Fto) gene: fasting affects Fto expression

A. Tiwari, S.M. Krzysik-Walker, R. Ramachandran*

Department of Poultry Science, the Pennsylvania State University, University Park, PA 16802

Received 10 May 2011; received in revised form 19 August 2011; accepted 21 August 2011

Abstract

Fat mass and obesity associated gene (Fto), also known as *Fatso*, is a member of the Fe–II and 2-oxoglutarate-dependent dioxygenase superfamily. Recent studies in humans and rodents suggest that Fto is involved in food intake regulation and lipid metabolism, whereas single nucleotide mutations in the Fto gene are associated with obesity and type 2 diabetes. The Fto gene is highly conserved from green algae to humans, but little is known about the avian Fto gene or protein. The objectives of the current study were to clone full-length chicken Fto cDNA and to determine the effect of age or feeding status on Fto expression. With the use of rapid amplification of cDNA ends, the full-length chicken Fto cDNA was cloned and found to share 63% to 66% homology with the mammalian Fto nucleotide sequence. Several regions of the chicken Fto protein, including the substrate (2-oxoglutarate) binding domains, were found to be identical to mammalian Fto protein. Western blotting with anti-human Fto antibody and reverse transcription PCR studies showed that Fto protein and gene were ubiquitously expressed in various tissues of the chicken. With the use of quantitative PCR, Fto mRNA levels were found to be higher in liver and skeletal muscle of 8-wk-old chickens than in 4-wk-old chickens. In addition, alterations in feeding status resulted in significant changes in Fto mRNA and Fto protein expression in the liver but not in skeletal muscle and adipose tissue of broiler chickens. Taken together, our data suggest that Fto probably plays a significant role in liver function and energy metabolism in the chicken.

Keywords: Liver; Skeletal muscle; Adipose tissue; Energy homeostasis; Age; Body growth

1. Introduction

Fat mass and obesity associated gene (Fto) has gained attention recently because of its possible role in obesity, hypertension, and other metabolic diseases. Several studies have associated single nucleotide polymorphisms in the Fto gene with childhood and adult obesity [1,2], polycystic ovarian syndrome [3], and type 2 diabetes[4]. First identified as *Fatso* in fused toes mutant mice because of transgene integration into the region D of chromosome 8 [5], this large gene consists of 350 kb of nucleotides, spans more than nine exons [6,7] and is highly conserved across vertebrate species [8]. Fto is classified as a member of Fe (II) and 2-oxogluterate dependent dioxygenases superfamily [8], members of which are involved in DNA repair, post-translational modification of proteins, and fatty acid metabolism [9]. Localized to the nucleus [6,8], Fto is involved in demethylation of 3-methyl thymine and 3-methyl uracil in single-stranded DNA and RNA [8,10]. In addition, mouse Fto has been suggested to act as a transcriptional coactivator in the regulation of adipose tissue development and maintenance [11].

* Corresponding author at: Department of Poultry Science, the Pennsylvania State University, University Park, PA 16802, Tel.: 814 865 5202; fax: 814 865 5691.

E-mail address: rameshr@psu.edu (R. Ramachandran).

0739-7240/12/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.domaniend.2011.08.001

Recent rodent studies have suggested a role for Fto in the regulation of food intake. A higher level of Fto expression has been reported in regions of the brain associated with food intake and energy expenditure in mammals [8,12,13]. Deletion of the Fto gene resulted in elevated sympathetic activation and greater energy expenditure in mice [14]. $Fto^{-/-}$ mice were also hyperphagic and lean compared with their $Fto^{+/+}$ littermates [14]. Targeted overexpression of the Fto gene in hypothalamic arcuate nucleus of rats decreased feed intake, whereas reducing arcuate Fto expression increased feed intake [15]. The role of Fto in feed intake, however, is not unequivocal, because a recent study suggested that Fto overexpression increases feed intake in mice on both standard and high-fat diets [16]. In addition to feed intake, Fto probably affects energy homeostasis by promoting fat cell lipolysis [17]. A recent study found that Fto expression decreased during differentiation of human preadipocytes in vitro [18].

Although the physiological functions of Fto are being explored in humans and rodents, there are very few studies investigating Fto expression in nonmammalian species. The chicken provides an excellent model in which to investigate the role of Fto in feed intake and energy expenditure because of their rapid growth and high feed consumption. Broiler chickens have undergone genetic selection for rapid growth to occur from hatch until market weight in approximately 42 d. During this period, broiler chickens voraciously eat approximately 4.1 kg of feed to achieve a 40-fold increase in body weight arising from increases in skeletal muscle mass [19], primarily in the breast (pectoralis) muscle, as well as in visceral adipose tissue [20]. The Fto gene, therefore, may play an important role in energy metabolism in chickens. The objectives of the current study were to clone and characterize the nucleotide sequence of Fto mRNA and to determine whether Fto gene and protein expressions are influenced by feeding status and age in the broiler chicken.

2. Materials and methods

2.1. Animals

Commercial broiler chickens (Cobb) were maintained at the Poultry Research and Education Center of the Pennsylvania State University and exposed to a photoperiod of 16 h of light and 8 h of dark. The chickens were provided with water and feed ad libitum unless otherwise indicated. Broiler chickens undergo rapid body growth between 4 and 8 wk of age; therefore, chickens of 4- and 8 wk of age were selected (n = 5 per age) to examine the effect of age on Fto expression. To determine the influence of feeding status on Fto expression, chickens (8 wk old) were fed ad libitum, fasted for 48 h, or fasted for 48 h followed by refeeding for 24 h (n = 5 chickens per treatment). Chickens were euthanized by decapitation, and selected tissues were collected, frozen in liquid nitrogen, and stored at −80 °C until further analysis by real-time quantitative PCR and Western blotting. All animal procedures were in accordance with protocol approved by the Institutional Animal Care and Use Committee.

2.2. Cloning and sequencing of full-length chicken Fto mRNA

Fto cDNA was cloned from total RNA extracted from chicken adipose tissue with the use of Trizol (Invitrogen, Carlsbad, CA, USA) as described earlier [21,22]. After DNase-I (Qiagen, Valencia, CA, USA) treatment, first-Strand cDNA was synthesized by reverse transcribing 1 μg of total RNA using $d(T)_{30}$**A/G/CA/G/C/T** primer and 2 U of Powerscript reverse transcriptase (Clontech, Palo Alto, CA, USA). Forward and reverse primers were developed from a highly conserved region of mammalian (human, mouse, and swine) Fto cDNA to amplify a partial cDNA-encoding chicken Fto mRNA. The PCR consisted of 300 nM of the forward and reverse primers (forward-**TGTAGGCTTGAAGGTAGCGTGGGACATA**; reverse-**ATTTCAAATACCGCTTGCCTTGAAACCA**), 300 nM of dNTP mixture (Invitrogen) and 2.5 U of deep $vent_R$ polymerase (New England Biolabs, Beverly, MA, USA). With the use of a DNA Engine PTC-200 thermal cycler (MJ Research, Reno, NV, USA), PCR was conducted with the following thermocycle: 94 °C for 1 min, 30 cycles of 94 °C for 5 sec, 58 °C for 10 sec, and 62 °C for 30 sec with a final extension of 62 °C for 10 min. The PCR product was gel purified and subcloned into pGEM-T Easy vector (Promega, Austin, TX, USA), and both sense and antisense strands were sequenced with Sp6 and T7 primers (Davis Sequencing, Davis, CA, USA). Chicken Fto gene-specific primers were designed from the above partial cDNA sequences for 5′/3′ rapid amplification of cDNA end (RACE) reactions with the use of a SMART RACE kit (Clontech). The 5′ and 3′ ends of the chicken Fto cDNA were then amplified, following manufacturer's instructions, and subcloned into pGEM-T Easy (Promega) and sequenced. The nucleotide and protein sequences of chicken and mammalian Fto were then compared with the use of ClustalW (v1.83) multiple sequence align-

ment tool in MacVector 11.0 program (MacVector, Cary, NC, USA).

2.3. Reverse transcription PCR

Total RNA was extracted from various tissues [hypothalamus, pituitary, heart, spleen, kidney, testes, liver, adipose tissue, skeletal (pectoralis) muscle] of chickens with the use of Trizol (Invitrogen) and RNeasy kit (Qiagen) as described earlier [21]. Total RNA was treated with DNase-I (Qiagen) on RNeasy column for degrading genomic DNA. First-Strand cDNA was prepared by reverse transcription (RT) with the use of 1 μg of total RNA, random primer 12 (New England Biolabs), Moloney murine leukemia virus reverse transcriptase (New England Biolabs), RNase out (Invitrogen), and dNTP mix (Roche Applied Science, Indianapolis, IN, USA) in a 20-μL reaction. To determine expression of the Fto gene in various tissues (hypothalamus, pituitary, heart, spleen, kidney, testes, liver, adipose tissue, skeletal muscle (pectoralis)], RT-PCR was performed with 50 ng single-stranded cDNA (from each tissue) as template, dNTP (Roche Applied Science), Taq polymerase (New England Bio-Lab) and (forward-TGTAGGCTTGAAGGTAGCGTGGGACA-TA; reverse-ATTTCAAATACCGCTTGCCTT-GAA-ACCA) with the following thermocycle: 94 °C for 1 min, followed by five cycles of 94 °C for 5 sec, 72 °C for 3 min, and 94 °C for 5 sec in DNA engine (MJ Research). For negative controls, RT reactions with the use of 1 μg of total RNA from each tissue with no reverse transcriptase were used as a template in place of RT reactions that contained reverse transcriptase. PCR products were separated on 1% agarose gel by electrophoresis and visualized in ethidium bromide staining under ultraviolet light.

2.4. Real-time quantitative PCR

To determine the effect of feeding status or age on Fto mRNA levels, a real-time quantitative PCR (qPCR) was performed. The Fto mRNA levels in liver, adipose tissue (abdominal fat pad), and skeletal muscle (pectoralis) were quantified in 7,500 Fast-Real Time PCR System (Life Technologies, Carlsbad, CA, USA). One microgram of total RNA was reverse transcribed as described above. The qPCR mixture was prepared by combining 50 ng of single-stranded cDNA, 1× PerfeCTa SYBR Green Fastmix (Quanta Biosciences, Gaithersburg, MD, USA), and 300 nM of forward and reverse primers to amplify Fto, chicken β-actin, or chicken 18S with the use of the following oligonucleotide primers: Fto forward, 5′-GACATAGAGACAC-CTGGATTAGCAA-3′, and reverse,5′-AATATGTAT-TCCAATGTTCCTCTTGAA-3′; chicken β-actin forward,5′-CTGGCACCTAGCACAATGAA-3′, and reverse, 5′-CTGCTTGCTGATCCACATCT-3′; chicken 18S forward,-5′-GTATGGTTGCAAAGCTGAAACT-TA-3′, and reverse, 5′-AAGAGCTCTCAATCTGTC-AATCCT-3′,). Thermocycle parameters were 95 °C for 20 sec followed by 35 cycles of 95 °C for 3 sec, 35 °C for 10 sec, 63 °C for 30 sec, 95 °C for 15 sec, and 60° for 1 min. At the end of amplification, a melting curve analysis was done by heating the PCR products 60 °C to 95 °C and held for 15 sec at increments of 0.2 °C, and the fluorescence was detected to confirm the presence of a single amplification product. Tissue samples from each animal were run in duplicate to obtain average C_T values for Fto mRNA and 18S mRNA (skeletal muscle) or β-actin (liver and adipose tissue). The log-linear threshold values (C_T) during the exponential phase of the PCR for Fto mRNA were subtracted from that of 18S mRNA for skeletal muscle or β-actin mRNA for liver and adipose tissue. The Fto mRNA quantity was expressed as a proportion of the respective housekeeper quantity following the $2^{-\Delta\Delta C}{}_T$ method for converting log-linear C_T values to linear term [23]. The relative amounts of Fto mRNA in the various tissues were then compared.

2.5. Protein extraction and immunoblotting analysis

Approximately 0.2 to 0.3 g of liver, adipose tissue, skeletal muscle, hypothalamus, pituitary, heart, spleen, kidney, or testes were homogenized with Tekmar Tissumizer (Tekmar, Co, Cincinnati, OH, USA) in 2 mL of RIPA lysis buffer [10 mM Tris-HCl, 150 mM NaCl (pH 8.0), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] containing protease inhibitor and phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich, St Louis, MO, USA) as described earlier [24]. Tissue homogenate was then passed through a 22-gauge needle for 10 times and shaken in a thermo-mixer (Eppendorf, Westbury, NY, USA) at 1,000 rpm for 30 min at 4 °C. The tissue lysate was centrifuged at 14,000 × g for 15 min at 4 °C, and supernatant fluid was collected. Total protein concentration was estimated by a protein dye-binding assay [25] with the use of a commercial kit (Bio-Rad, Hercules, CA, USA) with chicken ovalbumin as the standard. Aliquots of protein extracts were stored at −80 °C until analyzed by Western blotting.

Immunoblotting analyses were performed under reducing conditions with the use of the NuPAGE Novex bis-Tris minigel and XCell SureLock minicell (Invitrogen, Carlsbad, CA, USA) according to manufacturer's

recommendations. Samples were prepared by combining approximately 10 μg of total tissue protein extract with NuPAGE LDS sample buffer (4×) and NuPAGE reducing agent (10×) and heating for 10 min at 70 °C. Electrophoresis was performed on a 10% bis-Tris polyacrylamide gel (Invitrogen) with the use of 3-(*N*-morpholino)propanesulfonic acid running buffer under reducing conditions and electrotransferred onto Immun-Blot polyvinylidene difluoride membranes (0.2 μm; Bio-Rad). Membranes were incubated in blocking buffer (5% nonfat dry milk; Cell Signaling Technology, Boston, MA, USA) in Tris-buffered saline with 10% Tween 20 (Bio-Rad) for 2 h at room temperature. Membranes were then incubated with an affinity-purified rabbit anti-human Fto antibody (Novus Biologicals, Littleton, CO, USA; 0.275 μg/mL) in blocking buffer overnight at 4 °C with gentle agitation. The primary antibody used in this study was raised against a human partial Fto protein sequence in the C-terminal region (amino acids 400 to 505) that was approximately 75% homologous to the corresponding peptide sequence of chicken Fto. After several washes, membranes were incubated in horseradish peroxidase–labeled goat anti-rabbit IgG (Pierce, Rockford, IL, USA; 0.08 μg/mL) in blocking buffer for 1 h at room temperature with gentle agitation. Membranes were then treated with ECLPlus Chemiluminescence Detection Reagent (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's recommendations. Chemiluminescent signals were detected with the Storm 860 Optical scanner (Amersham Biosciences) and analyzed with Image Quant TL software (Amersham Biosciences). The chemiluminescence signal intensity of each band was calculated with a local average background correction. To normalize for protein load, membranes were reprobed with mouse anti-α-tubulin antibody (Sigma-Aldrich; 0.7 μg/mL) in blocking buffer at 1:1,428 dilution followed by incubation in horseradish peroxidase–labeled goat anti-mouse IgG (Pierce; 0.08 μg/mL) in blocking buffer. The chemiluminescent signal was then detected as described previously. The Fto protein quantity was expressed as a proportion of α-tubulin, and relative amounts of Fto protein were then compared.

2.6. Statistical analyses

The effect of fasting and refeeding on Fto mRNA and protein levels in liver, adipose tissue, or skeletal muscle were compared by a one-way ANOVA test with the use of SigmaPlot 11 (SYSTAT Software, San Jose, CA, USA). The Fto mRNA and protein levels in liver, adipose tissue, and skeletal muscle in 4- and 8-wk-old chickens were compared with Student *t*-test with the use of SigmaPlot 11. A probability level of $P < 0.05$ was considered statistically significant.

Table 1
Similarity of chicken Fto to mammalian Fto.

Species	GenBank Accession number	Nucleotide similarity, %	Amino acid similarity, %
Human	NM_001080432	66.4	72.5
Cow	NM_001098142	65.5	73.1
Mouse	BC022222	64.2	72.3
Rat	AM233906	63.7	72.1
Pig	EU249758	64.3	71.9

3. Results

3.1. Cloning of chicken Fto cDNA

The full-length Fto cDNA sequence was cloned from chicken adipose tissue, and the nucleotide sequences have been deposited into GenBank (accession number awaited). The open reading frame of chicken Fto cDNA consisted of 1,524 nucleotides with a predicted protein of 507 amino acids. The chicken Fto cDNA and its deduced protein sequences shared identities of 63% to 66% and 72% to 73%, respectively, to human, cow, mouse, rat, and pig Fto sequences (Table 1). Several regions of chicken Fto protein sequence showed a higher degree of homology (Fig. 1) to mammalian Fto sequences. In particular, the substrate (2-oxoglutarate) binding domains were identical in both chicken and mammalian Fto proteins.

3.2. Characterization of Fto gene and protein expression

Reverse transcription PCR analysis with the use of cDNA from chicken tissues showed that the Fto gene is expressed in adipose tissue, liver, skeletal muscle, hypothalamus, pituitary, heart, kidney, testes, and spleen (Fig. 2I). With the use of Western blotting, the Fto protein with a predicted mass of 58.7 kDa was detected in the chicken liver, skeletal muscle, adipose tissue, hypothalamus, spleen, kidney, heart, and testes (Fig. 2II). The chicken Fto protein was deduced from an immunoreactive band on the basis of estimated molecular weight. Additional bands of approximately 42 or 27 kDa were also observed, which may represent alternatively spliced or truncated forms of the Fto protein. The negative control, in which the primary antibody was omitted, did not yield any immunoreactive bands (data not shown).

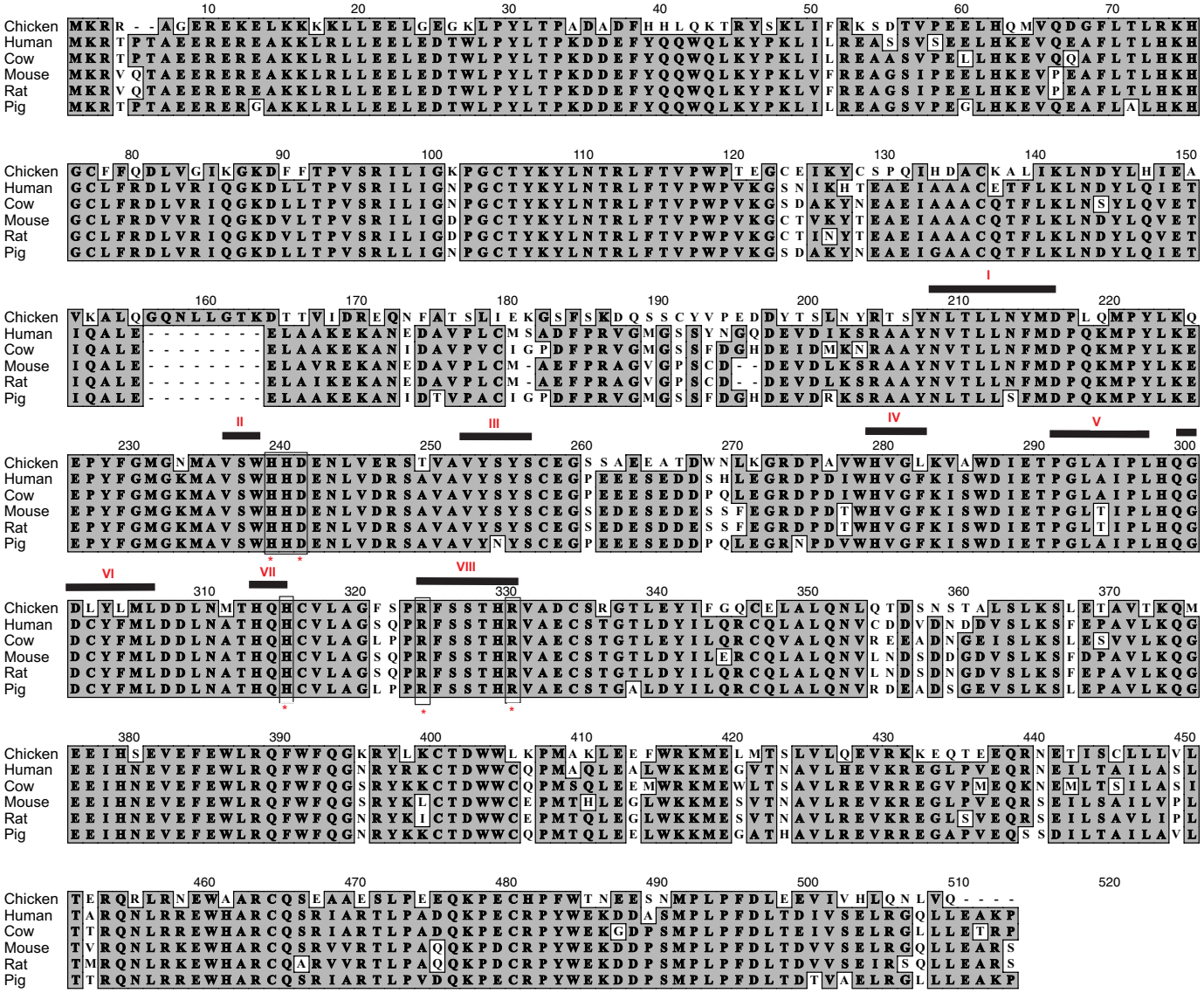

Fig. 1. Multiple sequence alignment of Fto from chicken, human, cow, rat, mouse, and pig. Sequence of the chicken Fto protein was deduced from Fto cDNA amplified from adipose tissue. Mammalian and chicken Fto sequences were aligned with the use of the ClustalW program. Conserved amino acid residues are shaded. Solid bars with roman numerals identify eight β strands that form the conserved double-stranded beta-helix of Fto that is characteristic of 2-oxoglutarate oxygenases[8]. Amino acids highlighted with asterisks [histidine (H^{239} and H^{315}), aspartatic acid ($D,^{241}$ and arginine (R^{320} and R^{324})] are the conserved domains for ligand (2-oxoglutarate) binding. Mammalian Fto protein sequences were deduced from respective Fto cDNA sequences obtained from the National Center for Biotechnology Information database with the accession numbers provided in Table 1.

3.3. Effect of age on tissue-specific Fto mRNA and protein levels

To analyze the effect of age on Fto expression, total RNA and protein were extracted from liver, skeletal muscle, and adipose tissue from 4- and 8-wk-old chickens, representing a rapid growth phase of doubling in body weight. Real-time qPCR analysis showed that Fto mRNA expression was significantly higher in liver ($P \leq 0.006$; n = 5) and skeletal muscle ($P \leq 0.008$; n = 5) of 8-wk-old chickens compared with 4-wk-old chickens (Fig. 3I-II). Adipose tissue Fto mRNA expression did not differ between the two age groups (Fig. 3III; $P > 0.05$). Similarly, Fto protein levels in liver, skeletal muscle, and adipose tissue were not statistically significant between 4- and 8-wk-old chickens ($P > 0.05$).

3.4. Effect of fasting and refeeding on Fto mRNA and protein expression

Feed deprivation for 48 h resulted in a significant increase in Fto mRNA expression in liver (Fig. 4I, $P \leq 0.001$). Refeeding for 24 h after 48 h fasting decreased Fto mRNA levels compared with the 48 h fasting ($P \leq 0.01$), thus restoring liver Fto mRNA levels to those observed in chickens fed ad libitum. Western blot analyses showed that liver

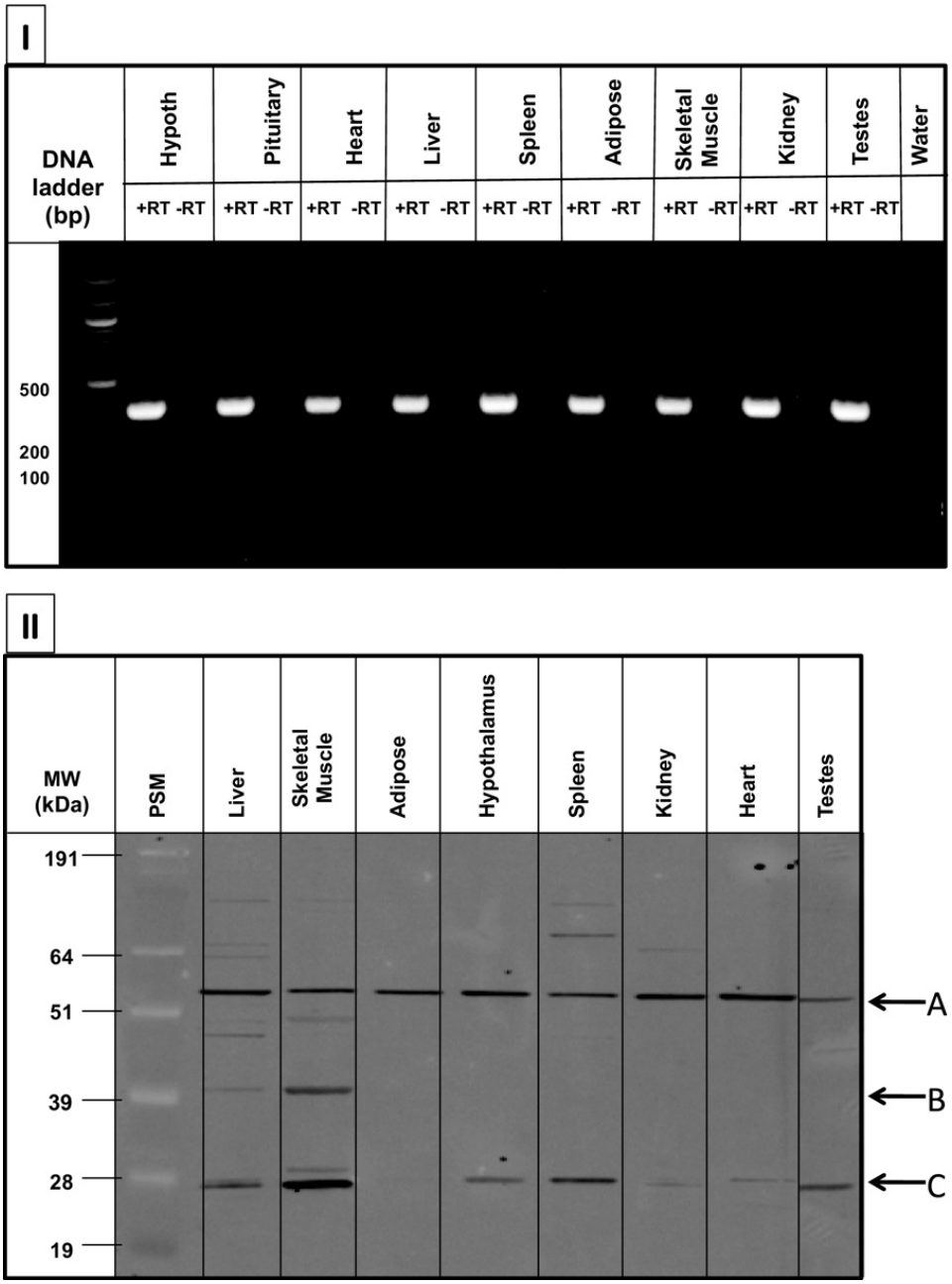

Fig. 2. Fto gene (I) and protein (II) expression in various tissues of the chicken. Total RNA extracted from each tissue was DNAse-I digested and reverse transcribed. Approximately 50 ng of cDNA (+RT) was used as template to amplify a 350-bp partial Fto cDNA. Negative control included RNA from each tissue without reverse transcriptase (−RT) and use of nuclease-free water in place of template cDNA. Tissue protein extracts were subjected to Western blot analysis under reducing conditions. Chicken Fto protein (A) was detected with anti-human Fto antibody and chemiluminescence. B and C represent possible splice variants of Fto protein. MW, -molecular weight; PSM, -prestained marker; Hypoth, hypothalamus.

Fto protein levels were also significantly elevated in response to fasting, whereas refeeding reduced these levels significantly compared with the fasting group ($P \leq 0.05$). In contrast to liver, skeletal muscle Fto mRNA levels did not differ ($P > 0.05$) among fed, fasted, and fasted and refed states (Fig. 4II). Skeletal muscle Fto protein level did not differ in response to 48 h fasting, although refeeding for 24 h did significantly reduce skeletal muscle Fto mRNA levels compared with fasted animals ($P \leq 0.005$). In addition, adipose tissue Fto mRNA and protein levels did not differ among animals that were fed ad libitum, 48 h fasted, and 48 h fasted followed by 24 h refeeding (Fig. 4III; $P > 0.05$).

4. Discussion

This is the first report of cloning and characterization of chicken Fto cDNA and expression of Fto gene and protein. A partial cDNA encoding chicken Fto

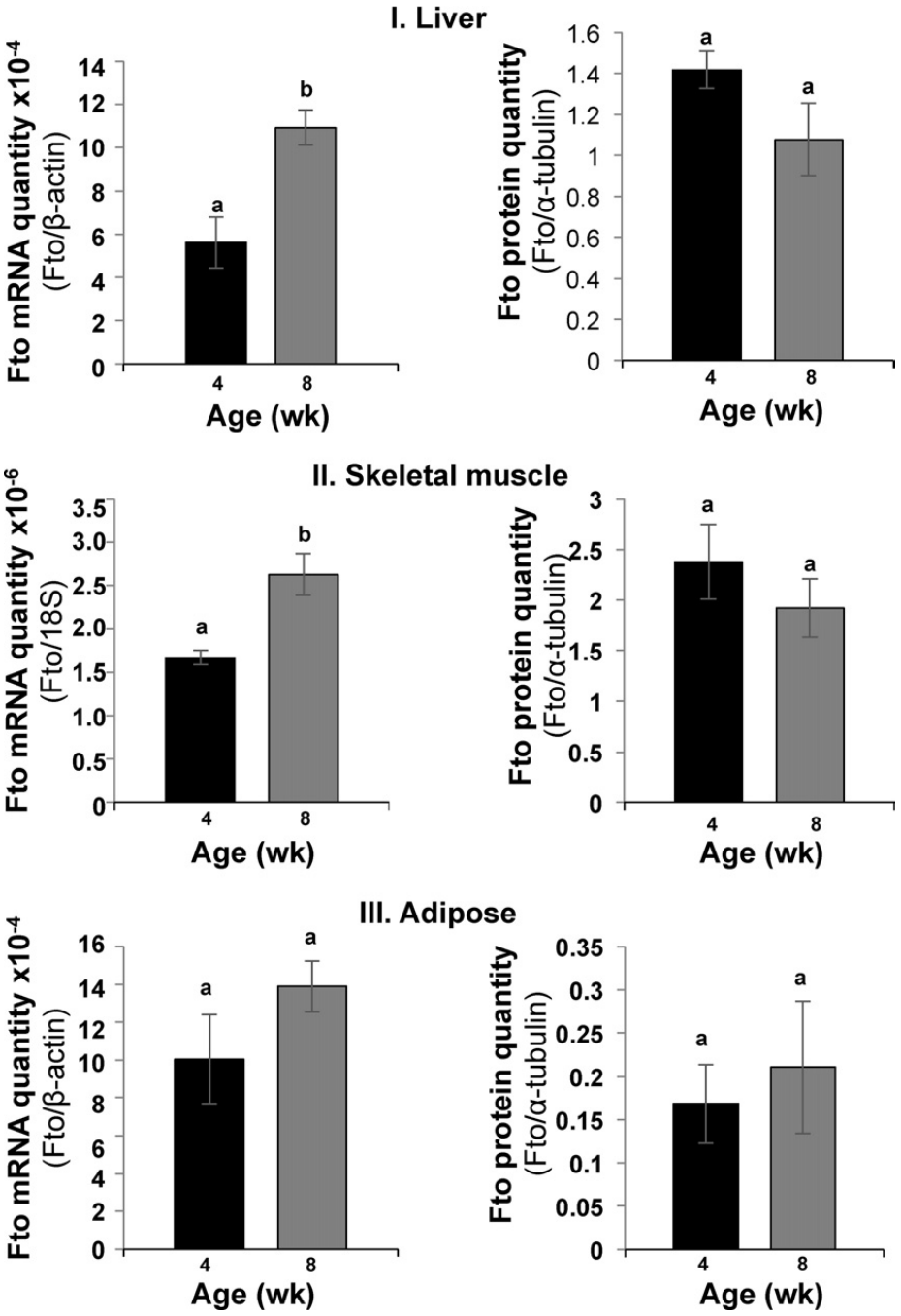

Fig. 3. Effect of age on Fto mRNA and protein expression in liver (I), skeletal muscle (II), and adipose tissue (III). To examine the effect of age on Fto mRNA or protein expression, tissues from 4- and 8-wk-old chickens (n = 5/age group) were collected for RNA and protein extraction. Approximately 50 ng of cDNA from each tissue was used as template in real-time quantitative PCR with the use of SYBR green as the dye to quantify chicken Fto mRNA levels relative to β-actin or 18S mRNA levels in separate reactions. Each reaction was run in duplicate, and threshold (C_T) values for Fto mRNA were subtracted from that of endogenous control (β-actin mRNA levels for liver and adipose tissue and 18S mRNA levels for skeletal muscle) and converted from log-linear to linear term. Approximately 10 μg of tissue protein extracts were subjected to immunoblot analysis under reducing conditions. Fto and α-tubulin were detected with anti-human Fto antibody and mouse anti-α-tubulin antibody, respectively, followed by chemiluminescence. Immunoreactivity was analyzed by densitometric quantification. Data with different letters above each bar within each chart represents significant difference at $P \leq 0.05$.

(Accession number EU751607) as well as the coding region of the chicken Fto mRNA (Accession number NM_001185147) were found to be identical to the corresponding regions of the full-length chicken Fto cDNA reported herein. The Fto gene is highly conserved from green algae to humans [26], and our data suggest that several regions of the chicken Fto cDNA and protein sequences exhibit considerable homol-

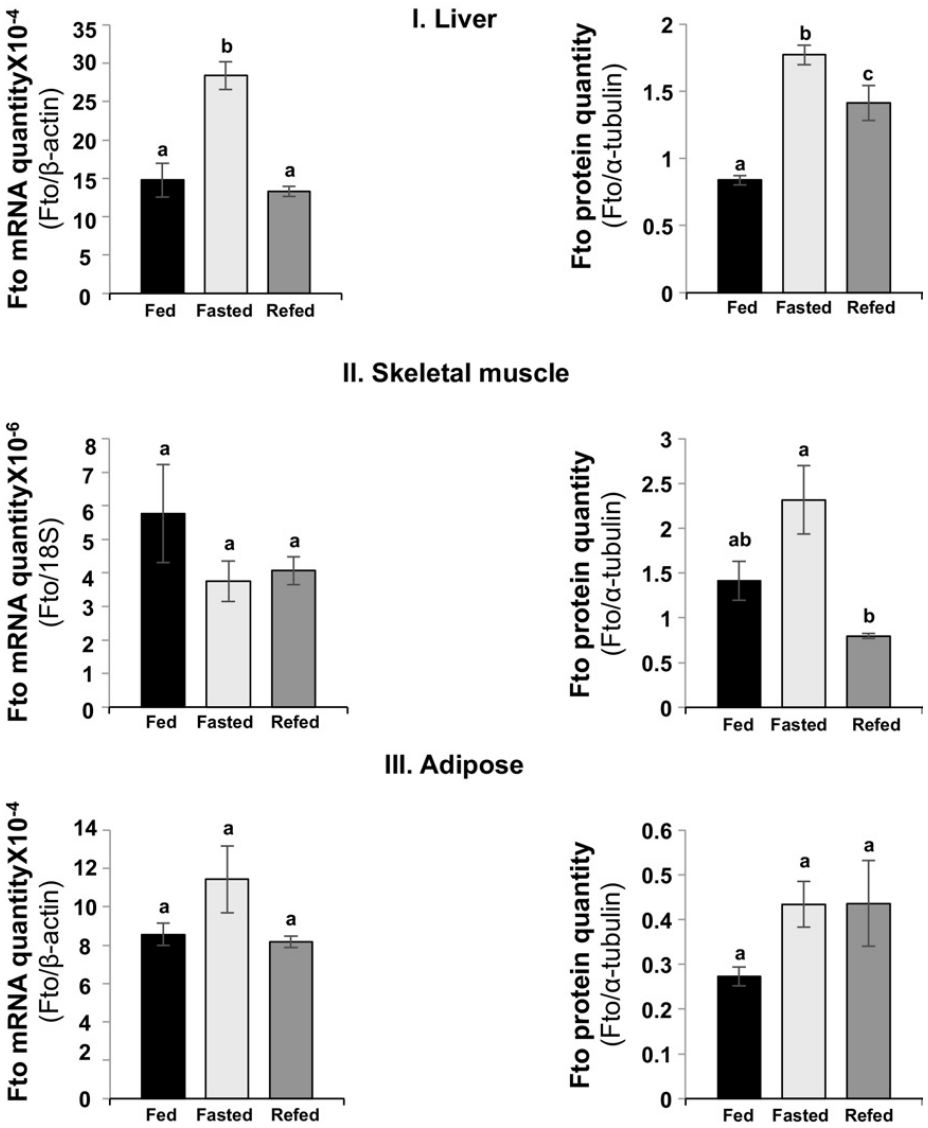

Fig. 4. Effect of feeding status on Fto mRNA and protein expression in the chicken liver (I), skeletal muscle (II), and adipose tissue (III). To examine the effect of feeding status on Fto mRNA and protein expression, tissues from fed, fasted, and refed chickens (n = 5/status) were collected for quantification of Fto mRNA and protein as described in 2. Materials and methods. Data with different letters above each bar represents significant difference at $P \leq 0.05$.

ogy to mammalian Fto sequences. Similar to mammalian Fto, chicken Fto contains a double-stranded beta-helix fold as in Fe(II) and 2-oxoglutarate oxygenases [8]. In particular, the residues that bind to 2-oxoglutarate, such as His^{239}, Asp^{241}, His^{315}, as well as Arg^{320} and Arg^{324}, in the predicted double-stranded beta-helix fold are conserved in the chicken and mammalian Fto protein sequences [26,27]. This conservation in the substrate binding domain suggests that chicken Fto probably has similar enzymatic functions as in mammals. Consistent with previous mammalian reports [1,2,5], the current study found that the Fto gene and protein are expressed in various tissues in the chicken. Ubiquitous expression of Fto suggests that it is required for critical cellular function(s), although the exact physiological relevance is currently unknown. As a member of the 2-oxoglutarate dioxygenase superfamily, Fto is probably involved in demethylation and nucleic acid repair, post-translational modification of proteins, and lipid metabolism [9].

To determine the effect of age on Fto expression, Fto mRNA and protein levels were quantified in the primary metabolic tissues of 4- and 8-wk old chickens. The present study found that the Fto mRNA levels were significantly higher in liver and skeletal muscle of 8-wk-old chickens than in 4-wk-old chickens. The increase in liver and skeletal muscle Fto mRNA levels

with age may be because of the rapid growth of skeletal muscle in chickens, which have been selectively bred for enlarged breast (pectoralis) muscle, body weight gain, and feed intake. Although Fto mRNA levels in the liver and skeletal muscle were significantly elevated, no difference at the protein level was observed between the two age groups, suggesting increased turnover of Fto protein or diminished translation of Fto mRNA into Fto protein.

The role of feeding status on Fto expression was examined in chickens placed on three different feeding regimes. Liver Fto mRNA levels were significantly increased after feed deprivation for 48 h, whereas refeeding decreased Fto mRNA levels back to levels similar to those of chickens fed ad libitum. Confirming the fasting-induced increase in liver Fto mRNA levels, Fto protein levels were also elevated. Refeeding resulted in a significant reduction in liver Fto protein levels compared with fasting, although these levels remained significantly higher than that observed in the ad libitum fed state. Our findings are consistent with a recent report that a 30-h fasting in mice significantly elevated liver Fto mRNA expression [28]. Fto has been reported to be associated with lipolysis in human adipocytes; [17] thus, fasting may lead to a deprivation of energy through lipolysis in the liver. The increase in liver Fto expression in fasted chickens may consequently promote lipolysis in an attempt to restore energy balance, because liver is the primary tissue for lipid metabolism in avian species [29]. In support of hypothesis, liver Fto levels decreased in response to refeeding, during which energy demands would be primarily met by the dietary feed intake rather than lipolysis. Contrary to the lipolytic effect of Fto in the liver, a recent study suggests that Fto overexpression in skeletal muscle leads to lipogenesis in humans with type 2 diabetes [30]. Such tissue-specific roles for Fto in lipolysis/lipogenesis may exist and may require further investigation. Interestingly, broiler chickens that are genetically selected for rapid growth and hyperphagia were found to have greater hypothalamic Fto mRNA levels compared with leghorn chickens, which are selected for egg production and eat far less than the broiler chickens [31]. Taken together, Fto probably influences feed intake besides affecting liver functions in the chicken. Our data indicate that feeding status did not alter Fto mRNA and protein levels in adipose tissue and skeletal muscle. Consistent with our finding, fasting did not significantly affect Fto mRNA expression in mouse white or brown adipose tissues [28]. Nevertheless, fasting mice for 30 h increased Fto mRNA expression in skeletal muscle [28], indicating that muscle type or species or both may influence expression of Fto mRNA.

In summary, the current study identified that the chicken Fto gene and protein share significant homology with mammalian Fto sequences and is ubiquitously expressed. In addition, liver Fto expression is influenced by fasting and refeeding, suggesting that Fto probably plays a role in energy metabolism in the chicken. Further studies are necessary to elucidate the role of Fto in feed intake and lipid metabolism in chickens.

Acknowledgments

This study was supported in part by a National Research Initiative Competitive Grant (2007-35206-17905) from the USDA, Cooperative State Research, Education and Extension Service.

References

[1] Frayling TM, Timpson NJ, Weedon MN, et al. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science 2007;316:889–894.

[2] Dina C, Meyre D, Gallina S, et al. Variation in FTO contributes to childhood obesity and severe adult obesity. Nat Genet 2007;39:724–726.

[3] Ewens KG, Jones MR, Ankener W, et al. Fto and mc4r gene variants are associated with obesity in polycystic ovary syndrome. PLoS ONE 2011;6:e16390.

[4] Scott LJ, Mohlke KL, Bonnycastle LL, et al. A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants. Science 2007;316:1341–1345.

[5] Peters T, Ausmeier K, Rüther U. Cloning of fatso (fto), a novel gene deleted by the fused toes (ft) mouse mutation. Mamm Genome 1999;10:983–986.

[6] Church C, Lee S, Bagg EA, et al. A mouse model for the metabolic effects of the human fat mass and obesity associated FTO gene. PLoS Genet 2009;5;e1000599.

[7] Fischer J, Emmerling C, Ruther U. On the history of fto. Obes Facts 2008;1:43–44.

[8] Gerken T, Girard CA, Tung YC, et al. The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. Science 2007;318:1469–1472.

[9] Clifton IJ, McDonough MA, Ehrismann D, Kershaw NJ, Granatino N, Schofield CJ. Structural studies on 2-oxoglutarate oxygenases and related double-stranded beta-helix fold proteins. J Inorg Biochem 2006;100:644–669.

[10] Jia G, Yang CG, Yang S, et al. Oxidative demethylation of 3-methylthymine and 3-methyluracil in single-stranded DNA and RNA by mouse and human FTO. FEBS Lett 2008;582:3313–3319.

[11] Wu Q, Saunders RA, Szkudlarek-Mikho M, Serna Ide L, Chin KV. The obesity-associated FTO gene is a transcriptional coactivator. Biochem Biophys Res Commun 2010;401:390–395.

[12] Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. Nature 2006;443:289–295.
[13] Lein ES, Hawrylycz MJ N; Ayres M, Bensinger A, et al. Genome-wide atlas of gene expression in the adult mouse brain. Nature 2007;445:168–176.
[14] Fischer J, Koch L, Emmerling C, et al. Inactivation of the FTO gene protects from obesity. Nature 2009;458:894–898.
[15] Tung YC, Ayuso E, Shan X, et al. Hypothalamic-specific manipulation of Fto, the ortholog of the human obesity gene FTO, affects food intake in rats. PLoS ONE 2010;5:e8771.
[16] Church C, Moir L, McMurray F, et al. Overexpression of Fto leads to increased food intake and results in obesity. Nat Genet 2010;42:1086–1092.
[17] Wåhlén K, Sjölin E, Hoffstedt J. The common rs9939609 gene variant of the fat mass- and obesity-associated gene FTO is related to fat cell lipolysis. J Lipid Res 2008;49:607–611.
[18] Tews D, Fischer-Posovszky P, Wabitsch M. Regulation of FTO and FTM expression during human preadipocyte differentiation. Horm Metab Res 2011;43:17–21.
[19] Scheuermann GN, Bilgili SF, Hess JB, Mulvaney DR. Breast muscle development in commercial broiler chickens. Poult Sci 2003;82:1648–1658.
[20] Hood RL. The cellular basis for growth of the abdominal fat pad in broiler-type chickens. Poult Sci 1982;61:117–121.
[21] Maddineni S, Metzger S, Ocón O, Hendricks G, 3rd, Ramachandran R. Adiponectin gene is expressed in multiple tissues in the chicken: Food deprivation influences adiponectin messenger ribonucleic acid expression. Endocrinology 2005; 146:4250–4256.
[22] Ramachandran R, Ocón-Grove OM, Metzger SL. Molecular cloning and tissue expression of chicken adipor1 and adipor2 complementary deoxyribonucleic acids. Domest Anim Endocrinol 2007;33:19–31.
[23] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta c(t)) method. Methods 2001;25:402–408.
[24] Krzysik-Walker SM, Ocón-Grove OM, Maddineni SR, Hendricks GL, 3rd, Ramachandran R. Is visfatin an adipokine or myokine? Evidence for greater visfatin expression in skeletal muscle than visceral fat in chickens. Endocrinology 2008;149: 1543–1550.
[25] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
[26] Aravind L, Koonin EV. The DNA-repair protein AlkB, EGL-9, and leprecan define new families of 2-oxoglutarate- and iron-dependent dioxygenases. Genome Biol 2001;2:RESEARCH 0007.
[27] Valegård K, van Scheltinga AC, Lloyd MD, et al. Structure of a cephalosporin synthase. Nature 1998;394:805–809.
[28] Poritsanos NJ, Lew PS, Mizuno TM. Relationship between blood glucose levels and hepatic Fto mRNA expression in mice. Biochem Biophys Res Commun 2010;400:713–717.
[29] Leveille GA, Romsos DR, Yeh Y, O'Hea EK. Lipid biosynthesis in the chick. A consideration of site of synthesis, influence of diet and possible regulatory mechanisms. Poult Sci 1975;54: 1075–1093.
[30] Bravard A, Lefai E, Meugnier E, et al. FTO is increased in muscle during type 2 diabetes, and its overexpression in myotubes alters insulin signaling, enhances lipogenesis and ROS production, and induces mitochondrial dysfunction. Diabetes 2010;60:258–268.
[31] Yuan L, Ni Y, Barth S, Wang Y, Grossmann R, Zhao R. Layer and broiler chicks exhibit similar hypothalamic expression of orexigenic neuropeptides but distinct expression of genes related to energy homeostasis and obesity. Brain Res 2009;1273: 18–28.