A novel transcript of MEF2D promotes myoblast differentiation and its variations associated with growth traits in chicken

Hongjia Ouyang [1, 2], **Jiao Yu** [3], **Xiaolan Chen** [3], **Zhijun Wang** [3], **Qinghua Nie** [Corresp. 3]

[1] College of Animal Science & Technology, Zhongkai University of Agriculture and Engineering, Guangzhou, China

[2] Guangdong Province Key Laboratory of Waterfowl Healthy Breeding, Guangzhou, China

[3] Department of Animal Genetics, Breeding and Reproduction, College of Animal Science, South China Agricultural University, Guangzhou, Guangdong, China

Corresponding Author: Qinghua Nie
Email address: nqinghua@scau.edu.cn

Background. Development of skeletal muscle is closely related to broiler production traits. The myocyte-specific enhancer binding factor (MEF) 2D gene (*MEF2D*) and its variant transcripts play important parts in myogenesis. **Methods.** To identify the transcript variants of chicken *MEF2D* gene and their function, this study cloned chicken *MEF2D* gene and identified its transcript variants from different tissue samples. The expression levels of different transcripts of *MEF2D* gene in different tissues and different periods were measured, and their effects on myoblast proliferation and differentiation were investigated. Variations in MEF2D were identified and association analysis with chicken production traits carried out. **Results.** Four novel transcript variants of *MEF2D* were obtained, all of which contained highly conserved sequences, including MADS-Box and MEF2-Domain functional regions. Transcript *MEF2D-V4* was expressed specifically in muscle, and its expression was increased during embryonic muscle development. The *MEF2D-V4* could promote differentiation of chicken myoblasts and its expression was regulated by *RBFOX2*. The single nucleotide polymorphism (SNP) g.36186C>T generated a TAG stop codon, caused MEF2D-V4 to terminate translation early, and was associated with several growth traits, especially on early body weight. **Conclusion.** We cloned the muscle-specific transcript of *MEF2D* and preliminarily revealed its role in embryonic muscle development.

A Novel Transcript of MEF2D Promotes Myoblast Differentiation and Its Variations Associated with Growth Traits in Chicken

Hongjia Ouyang[1, 3], Jiao Yu[2], Xiaolan Chen[2], Zhijun Wang[2], Qinghua Nie[2]*

[1]College of Animal Science & Technology, Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China.

[2]Department of Animal Genetics, Breeding and Reproduction, College of Animal Science, South China Agricultural University, Guangzhou, Guangdong, China. [3]Guangdong Province Key Laboratory of Waterfowl Healthy Breeding, Guangzhou 510225, China

*Corresponding author: Qinghua Nie (Tel. 86 20-85285759. Fax.86 20-85280740. E-mail: nqinghua@scau.edu.cn).

Running head: Chicken MEF2D promotes myoblast differentiation.

16 **Abstract**

17 **Background.** Development of skeletal muscle is closely related to broiler production traits. The
18 myocyte-specific enhancer binding factor (MEF) 2D gene (*MEF2D*) and its variant transcripts
19 play important parts in myogenesis.

20 **Methods.** To identify the transcript variants of chicken *MEF2D* gene and their function, this
21 study cloned chicken *MEF2D* gene and identified its transcript variants from different tissue
22 samples. The expression levels of different transcripts of *MEF2D* gene in different tissues and
23 different periods were measured, and their effects on myoblast proliferation and differentiation
24 were investigated. Variations in MEF2D were identified and association analysis with chicken
25 production traits carried out.

26 **Results.** Four novel transcript variants of *MEF2D* were obtained, all of which contained highly
27 conserved sequences, including MADS-Box and MEF2-Domain functional regions. Transcript
28 *MEF2D-V4* was expressed specifically in muscle, and its expression was increased during
29 embryonic muscle development. The *MEF2D-V4* could promote differentiation of chicken
30 myoblasts and its expression was regulated by *RBFOX2*. The single nucleotide polymorphism
31 (SNP) g.36186C>T generated a TAG stop codon, caused MEF2D-V4 to terminate translation
32 early, and was associated with several growth traits, especially on early body weight.

33 **Conclusion.** We cloned the muscle-specific transcript of *MEF2D* and preliminarily revealed its
34 role in embryonic muscle development.

35 **Keywords:** chicken; *MEF2D*; myoblast; variant transcripts; SNP

1. Introduction

Myocyte-specific enhancer binding factor 2 (MEF2) family is present widely in muscle cells. It plays an important role in the development, growth and maintenance of organisms through interacting with various genes in the calcineurin signaling pathway (Potthoff and Olson, 2007). MEF2 is a major regulator of myogenic genes expression, which can activate expression of various myogenic related genes, and interact with members of myogenic regulatory factors (MRFs) to regulate myogenesis (Molkentin et al., 1995; Desjardins et al., 2016). In vertebrates, the MEF2 family has four members, including MEF2A, MEF2B, MEF2C and MEF2D genes. MEF2 belongs to the MADS-Box family of transcription regulators. The N-terminal of the four MEF2 proteins all contain the highly conserved MADS-box domain and MEF2 domain. The structural difference among them is due mainly to the difference in C-terminal transcriptionally active regions (Molkentin et al., 1996; Black et al., 1998). Breitbart *et al*. first cloned *MEF2D* in humans, and found that it plays a key role in muscle development (Breitbart et al., 1993). As a member of MEF2 family, *MEF2D* has been reported that plays a key role in myogenesis. In *MEF2D* knockout mice, the differentiation of muscle cells in each muscle tissue was found to be inhibited (Bour et al., 1995; Lilly et al., 1995). The *MEF2D* has also been found to be involved in skeletal myogenesis, cardiac hypertrophic growth and proliferation of vascular smooth muscle cells (Ogawa et al., 2013; Hu et al., 2017; Li et al., 2017).

The chicken *MEF2D* gene has been cloned, but only one transcript has been reported (Caldwell et al., 2005). In humans and mice, multiple different transcripts of *MEF2D* gene have been found, and these transcripts can perform different functions (Ogawa et al., 2013; Sebastian et al., 2013). In this study, we aim to identify the variant transcripts of chicken *MEF2D* gene from different tissue samples, measure expression levels of these transcripts in various tissues and at different periods, and to study their roles in skeletal myogenesis.

2. MATERIALS AND METHODS

61 *2.1 Animals*

62 The fertilized eggs of Xinghua chicken in this experiment were purchased from a livestock
63 farm of South China Agricultural University (Guangzhou, China). They were hatched in a full-
64 automatic incubator. During the period from the 10th embryo age (E10) to the 1st day post-
65 hatching (P1), the breast muscle and leg muscle tissues of 20 chickens were collected each day
66 and stored at -80 °C. Five 7-weeks-old Xinghua female chickens were purchased from a
67 livestock farm of South China Agricultural University. Fifteen tissues (cerebrum, cerebellum,
68 hypothalamus, pituitary, heart, liver, spleen, lung, kidney, breast muscle, leg muscle,
69 subcutaneous fat, abdominal fat, muscular stomach and glandular stomach) of each chicken were
70 collected and stored at -80 °C.

71 *2.2 DNA samples*

72 The DNA samples were obtained from an F_2 resource population crossed from Xinghua and
73 White Recessive Rock (XH&WRR) as described previously (Lei et al., 2005). The population
74 consists of 17 full-sibling families, and 434 F_2 individuals (221 male and 213 female chickens)
75 with a detailed record of growth traits, carcass traits, and meat quality traits. Weight (body, semi-
76 eviscerated, eviscerated, breast muscle, leg muscle and abdominal fat pad) was measured in
77 grams using an electronic scale. The shank length, head width, breast width, breast depth, and
78 body length were measured with vernier caliper. The shank diameter was measured in the middle
79 of the shank with string and straightedge.

80 *2.3 RNA isolation, cDNA synthesis and quantitative real time PCR (qPCR)*

81 Total RNA of all tissues were isolated using Trizol reagent (Invitrogen, Carlsbad, CA,
82 USA), following the recommended manufacturer's protocol. The quality and quantity of RNA
83 samples were assessed by gel electrophoresis and a spectrophotometer (NanoDrop 2000c;
84 Thermo, Waltham, MA). The cDNA synthesis was performed with 1 μg of RNA for each sample

using a RevertAid^{TM} First Strand cDNA Synthesis Kit (Fementas, Waltham, MA, USA) in a total reaction volume of 20 μL.

The mRNA level of MEF2D and its four variant transcripts, RBFOX2, MHC and MYOD were measured by qPCR. The qPCR was performed using SsoFast Eva Green Supermix (BIO-RAD, Hercules, USA) in CFX9600 (BIO-RAD). Each sample was assayed in triplicate under the following conditions: 95 °C for 2 min, followed by 40 cycles of 10 s at 95 °C, 30 s at the annealing temperature (58-62 °C), 30 s at 72 °C, a melt curve by 65 °C to 95 °C, and increments 0.5 °C for 5 s. Chicken *GAPDH* was used as the reference gene for tissue-samples of 7-weeks-old chickens and myoblasts, whereas *18S rRNA* was used as the reference gene for embryonic muscle samples. The relative mRNA level in each sample was calculated using the comparative $2^{-\Delta\Delta Ct}$ (CT is threshold cycle; $\Delta\Delta Ct = \Delta Ct_{\text{target sample}} - \Delta Ct_{\text{control sample}}$) method (Livak and Schmittgen, 2001).

2.4 Gene cloning and sequences analysis

Referring to the *MEF2D* gene sequence in chicken (NM_001031600.3) reported by National Center for Biotechnology Information (NCBI), primers were designed to amplify *MEF2D* gene by PCR. Products of PCR were purified using an Agarose Gel DNA Extraction Kit (Takara, Osaka, Japan) and then cloned into the pMD-18T vector (Takara) according to the manufacturer's protocol. Positive clones were identified by PCR and then sequenced by Invitrogen Co. Ltd (Guangzhou, China).

The sequencing results were analyzed and compared with the chicken genome (Gallus_Gallus-5.0/Galgalgal5; http://genome.ucsc.edu/cgi-bin/hgBlat) andMEF2D sequence (NM_001031600.3). DNAStar software (DNASTAR, Madison, WI) was used to analyze the homology of the amino acid (AA) sequence of MEF2D between different species and the conserved regions of the sequence. The amino acid sequences of MEF2D from the other species were obtained from GenBank (Table S1 in file S1).

110 *2.5 Plasmid construction, cell culture and transfection*

111 The coding sequences of chicken *RBFOX2* and *MEF2D-V4* were amplified from cDNA of
112 chicken leg muscle using PCR, and then cloned into the pEGFP-C1 vector (Invitrogen) using the
113 *EcoRI* and *BamHI* restriction sites.

114 Chicken primary myoblasts were isolated from the leg muscle of chickens at 10-11
115 embryo age as described previously (Luo et al., 2014). Cells were maintained in RPMI-1640
116 medium (Gibco, Grand Island, NY) supplemented with 20% (v/v) fetal bovine serum (Gibco),
117 and 100 μg/mL penicillin/streptomycin (Invitrogen) at 37°C with 5% CO_2, humidified
118 atmosphere. Cells were seeded in 12-well plates with 1 mL per well at 10^5 cells/mL. When the
119 cells had grown to 70%-80% confluence, they were transfected with plasmids (1 μg/mL) of
120 *MEF2D* or *RBFOX2* or pEGFP-C1 vector control using lipofectamine 3000 reagent (Invitrogen)
121 according to the manufacturer's instructions.

122 *2.6 Cell proliferation assay*

123 After overexpressing *RBFOX2* and *MEF2D* genes in myoblasts for 48 h, respectively, cells
124 were collected and fixed with 70% ethanol overnight at -20°C. The fixed cells were collected by
125 centrifugation at 1000×g, washed once with PBS, and stained with 0.5 mL propidium iodide (PI)
126 dye solution (5 mg PI +0.1 mL Triton X-100 +3.7 mg EDTA +10 mL PBS), and then incubate
127 for 30 min at 4°C in the dark. After staining, cells were detected by BD FACSAriaII flow
128 cytometer (BD, Franklin Lakes, NJ). The results were analyzed by software ModFit Lt 4.1.

129 *2.7 Western blotting*

130 Proteins of transfected myoblasts were extracted using RIPA lysis buffer (Beyotime,
131 Shanghai, China) and the concentration was determined by a bicinchoninic acid (BCA) protein
132 assay kit (Beyotime). The primary antibodies MYOG (1:500 dilution; Biorbyt, Cambridge, UK)
133 and MHC (1:1000 dilution; DSHB, Iowa, USA) were using to measure the protein levels of

MYOG and MHC respectively by Western blotting as described previously (Ouyang et al., 2018). GAPDH (1:1000 dilution; Bioworld, Minnesota, USA) was used as the reference gene.

2.8 Primers

Primers were designed using primer premier 5 software (PREMIER Biosoft, Palo Alto, CA, USA) and synthesized by Bioengineering Co., Ltd. (Shanghai, China). Specific primer sequences are shown in Table S2 and Table S3 in File S1.

2.9 Identification and genotyping of SNPs

Variations in the coding sequences of chicken MEF2D were identified using PCR with primers PM1-PM9 in our F_2 resource population (XH&WRR). The locations of primers were shown in Figure S1. PCR was performed in 50 μL of a mixture containing 50 ng of chicken genomic DNA, 25 pmol of primers and 25 μL PCR Master Mix (Transgen, Beijing, China), and using the following protocol: 94 °C for 3 min, followed by 32 cycles of 30 s at 94 °C, 30 s at the annealing temperature (58-63 °C), 30 s at 72 °C and 72 °C for 5 min at last. Twenty DNA samples were selected randomly from the F_2 resource population (XH&WRR) for PCR using primers PM1-PM9. PCR products were sequenced by Bioengineering Co., Ltd. (Shanghai, China) and the results were then blasted with each other to identify variations. The special SNPs we were interested were genotyped by PCR and sequencing in all DNA samples of the F_2 resource population (XH&WRR).

2.10 Statistical analysis

SNP frequencies were calculated using the observed numbers of alleles for each SNP. SNP genotypes were tested for Hardy–Weinberg equilibrium (HWE) with the chi-square test. Association analysis of SNPs and fatness traits were performed using the General Linear Models Procedures of SAS 9.0 (SAS Institute Inc., Cary, NC, USA) using the following model:

$$Y_{ijkl} = \mu + S_i + G_j + H_k + F_l + e_{ijkl}$$

Where Y = the traits phenotypic values; μ = the overall population mean; S = the effect of gender; G = the effect of genotype; H = the effect of incubation batch; F = the effect of family; e = the random residuals.

Data on gene expression were analyzed using SPSS 21.0 (IBM,Amonk, NY, USA). The ANOVA was used to compare expression levels among different groups. All values are presented as means ± standard error of mean (S.E.M). The threshold for significance was set at $P < 0.05$ and for high significance at $P < 0.01$.

2.11 Animal Ethics

Animal experiments were handled in compliance and all efforts were made to minimize suffering. It was approved by the Animal Care Committee of South China Agricultural University (Guangzhou, People's Republic of China) with approval number SCAU#0014.

3. Results

3.1 Sequence alignment and phylogeny analysis of MEF2D

According to the information from NCBI database, the chicken MEF2D gene cDNA sequence (NM_001031600.3) is 4111 bp in length, the coding region is 715-2271nt, and it encodes 518 amino acids (NP_001026771.3). Blast with the chicken genome (GRCg6a/galGal6), this gene is located on chicken chromosome 25 (2,742,900-2,782,225), the full length of the gene is 39,326 bp, and it contains 10 exons and 9 introns.

The protein sequences of *MEF2D* in 10 species (*Gallus gallus*, *Meleagris gallopavo*, *Coturnix japonica*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Sus scrofa*, *Bos taurus*, *Danio rerio* and *Xenopus laevis*) were compared and analyzed by homologous clustering. The results showed that the protein sequences of *MEF2D* were highly conserved, and had conserved

domain of MADS-Box (2-57 AA) and MEF2-Domain (58-86 AA) in chicken and the other 9 species tested (Figure 1A). Phylogenetic tree clustering showed that 10 species were divided into four distinct groups: birds (*Gallus gallus*, *Meleagris gallopavo* and *Coturnix japonica*), mammals (*Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Sus scrofa* and *Bos taurus*), *Danio rerio* and *Xenopus laevis* (Figure 1B). Homology between chicken, turkey and quail was more than 96%. Homology among mammals (human, mouse, rat, pig and cow) was also very high, while the homology between zebrafish and frogs and other species was relatively low (Figure 1C).

3.2 Variant transcripts of chicken MEF2D

In this experiment, cDNA samples from liver, hypothalamus and muscle tissue at different stages were used as PCR templates to clone chicken *MEF2D* gene, and positively clone PCR products were detected by agarose gel electrophoresis (Figure 2A). Sequencing analysis of PCR products identified four novel variant transcripts (V1-V4) of *MEF2D* (Figure 2B). Compared with the transcript of *MEF2D* gene on NCBI, the transcript V1 (NCBI accession number: KY680649) was 3222 bp in length, had a deletion of 889 bp (1446-2334 nt), and was predicted to encode 251 AA. The transcript V2 (KY680650) was 3616 bp in length, had a deletion of 498 bp (1139-1636 nt) and was predicted to encode 353 AA. The transcript V3 (KY680651) was 4135 bp in length, had an insertion of 21 bp after exon 8 (1570 nt) and an AAC insertion at 1813 nt, and was predicted to encode 526 AA. The full length of the transcript V4 (KY680652) was 4132 bp, and a 21 bp is inserted after exon 8 (1570 nt), and was predicted to encode 526 AA. The complete DNA and protein sequences of these four variants are shown in supplementary file S2.

Comparative analysis of the amino acid sequences of the four novel*MEF2D* transcripts V1-V4 revealed that they contained conserved functional MADS-Box and MEF2-Domain. The position and sequence of exon 4 (87-132 AA) of the transcript V4 was different from that of the

other transcripts. This was the same as the variant transcripts found in humans and mice, and they also mutated in the amino acid sequence 87-132 (Figure S2).

3.3 Tissue specific expression of MEF2D

The expression of *MEF2D* transcripts in the different tissues of chickens was measured. Two deletion transcripts, *MEF2D-V1* and *MEF2D-V2* were barely expressed. The main transcripts expressed were *MEF2D-1* (the same transcription as reported by NCBI), *MEF2D-V3* and *MEF2D-V4*. *MEF2D-1* and *MEF2D-V3* were expressed widely in various tissues, and the relative expression levels in adipose tissue and brain tissue were higher, and in the liver and kidney were lower (Figure 3A and 3B). The transcript *MEF2D-V4* exhibited muscle-specific expression and was highly expressed in the heart, chest muscles and leg muscles, but its expression in other tissues was extremely low (Figure 3C).We measured expression level of *MEF2D-V4* in embryonic leg muscles, and found that the expression level of *MEF2D-V4* increased from E11 to E19. The expression level of *MEF2D-V4* increased significantly at E15 and E17, and it was stably expressed at E17 to E19 (Figure 4).

3.4 Novel transcript MEF2D-V4 promotes myoblast differentiation in chicken

The sequence of MEF2D-V4 was similar to that of the human variant transcript Mef2Dα2, expression of which was regulated by RBFOX2 and was required for muscle differentiation (Singh et al., 2014; Runfola et al., 2015). Thus, to explore the effects of MEF2D-V4 and RBFOX2 on muscle differentiation, the eukaryotic overexpression vector of *RBFOX2* and *MEF2D-V4* were constructed, and transfected into chicken myoblasts respectively. After 48h, the expression levels of *RBFOX2* and *MEF2D-V4* were measured by qPCR: both of these two vectors could induce overexpression of the corresponding genes effectively. Furthermore, overexpression of *RBFOX2* gene could also increased the expression level of the *MEF2D-V4* significantly (Figure 5).

After overexpressing *MEF2D-V4* and *RBFOX2* in chicken primary myoblasts, the cell cycle was detected by flow cytometry. Compared with the control group, the number of S phase cells was increased in overexpressed *MEF2D-V4* or *RBFOX2* group, but did not reach significant levels ($P > 0.05$; Figure S3). After overexpressing *MEF2D-V4* and *RBFOX2* for 48 h in myoblast differentiation, the mRNA level of *MYOG* and *MHC* was both increased ($P < 0.05$) in cells overexpressing *MEF2D-V4* or *RBFOX2*, whereas MYOD expression was not significantly different (Figure 6A). The expression of *MYOG* and *MHC* was also detected by Western blot. Protein levels of MYOG and MHC were also increased, in accordance with mRNA levels (Figure 6B).

3.5 SNPs identification and its association analysis with production traits

In the F_2 resource population (XH&WRR), 31 SNPs were identified in the full length chicken MEF2D DNA through PCR sequencing (Table 1), including one insertion/deletion, 14 synonymous mutations and 16 missense mutations. Interestingly, there was a T-C mutation at exon 9, g.36186C > T, generate a TAG stop codon, resulting in a change in the coding sequence and termination of translation in both MEF2D-1and MEF2D-V4. Therefore, we performed PCR amplification and sequencing on exon 9 to genotyp and carried out association analysis this SNP in the F_2 resource population (XH&WRR). Several growth traits were associated significantly with this SNP g.36186C > T, including first days, 7, 14, 21, 28 and 63 days of body weight, 42, 77 and 88 days of shank length, 42 and 56 days of shank diameter, and 0-4 weeks of average weight gain (Table 2). The dominant genotype of SNP g.36195C > T was the CC type, and the average early body weight of TT type individuals was lower than that of CC type individuals.

In addition, g.36094CAGIns/Del (another SNP site of exon 9) was associated with carcass traits in chickens (Table 3), including eviscerated weight (EW), leg muscle weight (LMW), abdominal fat pad weight (AFW) and small intestine length (SIL). The dominant genotype of g.36094CAGIns/Del was the Del/del type. EW and LMW of the Del/del type were

lower than that of the Ins/ins type.

4. Discussion

MEF2D gene is a member of the MEF2 family and plays a key role in myogenesis (Du et al., 2008; Nebbioso et al., 2009; Della et al., 2012). *MEF2D* gene has several transcripts in humans and mice, and among them, there are specific transcripts that can have different functions (Ogawa et al., 2013; Sebastian et al., 2013), but only one transcript sequence has been reported in chicken. Therefore, we cloned the variant transcript of the *MEF2D* gene from several different tissues of chicken, and obtained four novel transcripts. Blast analyses of their amino acid sequences revealed that they all contained the conserved functional regions MADS-Box and MEF2-Domain, which conformed to the structural characteristics of the MEF2 family.

The position and sequence of exon 4 of the transcript *MEF2D-V4* was different from that of the original transcript *MEF2D-1*, that is, the amino acid sequence 87-132 was different. The mutation region of this new transcript was similar to the variant transcript found in humans and mice, and they were both mutated in the amino acid sequence 87-132 region (Edmondson et al., 1994; Ornatsky et al., 1996; Nagar et al., 2017). The human variant transcript Mef2Dα2 is expressed specifically in muscle, and it can avoid inhibitory phosphorylation, recruit Ash2L to activate muscle-related genes, and promote muscle cell differentiation (Sebastian et al., 2013). Splice variations were also found in mice, producing two transcripts, *Mef2D1a* and *Mef2D1b*. *Mef2D1a* can promote the expression of *MYOG* gene by binding to its promoter, and such binding is regulated by glycosylation (Ogawa et al., 2013). We examined the expression patterns of these four novel transcripts, and found that *MEF2D-V4* was also expressed specifically in muscle of the heart, breast and leg. The function of muscle-specific genes is often related to muscle development and growth. During embryonic development, the expression level of *MEF2D-V4* in leg muscle was increased significantly in the late stage embryos, indicating that *MEF2D-V4* may play an important role in embryonic muscle development and growth.

279 Therefore, we studied further the function of *MEF2D-V4* in chicken primary myoblasts. It has
280 been reported in mice that RBFOX2 regulates alternative splicing of the *MEF2D* gene (Singh et
281 al., 2014; Runfola et al., 2015). We also found that chicken *RBFOX2* promotes the expression of
282 *MEF2D-V4*. Overexpression of *RBFOX2* and *MEF2D-V4* promoted the differentiation of
283 chicken myoblasts.

284 Studies have shown that SNPs of MEF2D gene could affect the production performance
285 of livestock and poultry animals. The MEF2D variants have been found to be highly correlated
286 with MEF2D mRNA and protein levels in the *longissimus dorsi* muscle of cattle (Juszczuk-
287 Kubiak et al, 2012). In duck, a CAG repeat polymorphism has been found in MEF2D gene. This
288 CAG repeat can generate significantly longer transcription products and positive correlations
289 with five muscle-related traits (Wang et al., 2016b). We also found that a CAG insertion/deletion
290 in MEF2D gene was associated with eviscerated weight and leg muscle weight of chicken.
291 Furthermore, g.36186C > T was found to be associated with body weight at 1, 7, 14, 21 and 28
292 days. This mutation generated a TAG stop codon, caused MEF2D-V4 to terminate translation early,
293 resulting in TT type individuals not being able to produce normal MEF2D-V4 protein products.
294 The average early body weight of TT type individuals was lower than that of CC type individuals,
295 which indicated that MEF2D V4 may be positively correlated with chicken growth traits and
296 promote early growth of chickens.

297 **5. Conclusions**

298 In summary, the *MEF2D* gene can produce the muscle-specific transcript *MEF2D-V4*,
299 which is positively regulated by *RBFOX2* and can promote the differentiation of chicken
300 myoblasts. Chicken *MEF2D* gene could regulate the embryonic development and early growth
301 of skeletal muscle by alternative splicing.

303 **References**

304 Black, B.L., and Olson, E.N. 1998. Transcriptional control of muscle development by myocyte
305 enhancer factor-2 (MEF2) proteins. *Annual Review of Cell and Developmental Biology*
306 14:167-196. 10.1146/annurev.cellbio.14.1.167
307 Bour, B.A., O'Brien, M.A., Lockwood, W.L., Goldstein, E.S., Bodmer, R., Taghert, P.H.,
308 Abmayr, S.M., and Nguyen, H.T. 1995. Drosophila MEF2, a transcription factor that is
309 essential for myogenesis. *Genes and Development* 9:730-741. 10.1101/gad.9.6.730
310 Breitbart, R.E., Liang, C.S., Smoot, L.B., Laheru, D.A., Mahdavi, V., and Nadal-Ginard, B.
311 1993. A fourth human MEF2 transcription factor, hMEF2D, is an early marker of the
312 myogenic lineage. *Development (Cambridge, England)* 118:1095-1106.
313 Caldwell, R.B., Kierzek, A.M., Arakawa, H., Bezzubov, Y., Zaim, J., Fiedler, P., Kutter, S.,
314 Blagodatski, A., Kostovska, D., Koter, M., Plachy, J., Carninci, P., Hayashizaki, Y., and
315 Buerstedde, J.M. 2005. Full-length cDNAs from chicken bursal lymphocytes to facilitate gene
316 function analysis. *GENOME BIOLOGY* 6:R6. 10.1186/gb-2004-6-1-r6
317 Della, G.B., Armand, A.S., Lecolle, S., Charbonnier, F., and Chanoine, C. 2012. Mef2d acts
318 upstream of muscle identity genes and couples lateral myogenesis to dermomyotome
319 formation in Xenopus laevis. *PLoS One* 7:e52359. 10.1371/journal.pone.0052359
320 Desjardins, C.A., and Naya, F.J. 2016. The Function of the MEF2 Family of Transcription
321 Factors in Cardiac Development, Cardiogenomics, and Direct Reprogramming. *Journal of*
322 *cardiovascular development and disease* 3.
323 Du M, Perry, R.L., Nowacki, N.B., Gordon, J.W., Salma, J., Zhao, J., Aziz, A., Chan, J., Siu,
324 K.W., and McDermott, J.C. 2008. Protein kinase A represses skeletal myogenesis by
325 targeting myocyte enhancer factor 2D. *MOLECULAR AND CELLULAR BIOLOGY* 28:2952-
326 2970. 10.1128/MCB.00248-08
327 Edmondson, D.G., Lyons, G.E., Martin, J.F., and Olson, E.N. 1994. Mef2 gene expression
328 marks the cardiac and skeletal muscle lineages during mouse embryogenesis.

DEVELOPMENT 120:1251-1263.

Hu, Z.Q., Luo, J.F., Yu, X.J., Zhu, J.N., Huang, L., Yang, J., Fu, Y.H., Li, T., Xue, Y.M., Feng, Y.Q., and Shan, Z.X. 2017. Targeting myocyte-specific enhancer factor 2D contributes to the suppression of cardiac hypertrophic growth by miR-92b-3p in mice. *Oncotarget* 8:92079-92089. 10.18632/oncotarget.20759

Juszczuk-Kubiak, E., Starzynski, R.R., Sakowski, T., Wicińska, K., Flisikowski, K. 2012. Effects of New Polymorphisms in the Bovine Myocyte Enhancer Factor 2D (MEF2D) Gene On the Expression Rates of the Longissimus Dorsi Muscle. *Molecular Biology Reports* 39:8387-8393. 10.1007/s11033-012-1689-6.

Li, K., Pan, J., Wang, J., Liu, F., and Wang, L. 2017. MiR-665 regulates VSMCs proliferation via targeting FGF9 and MEF2D and modulating activities of Wnt/beta-catenin signaling. *American Journal of Translational Research* 9:4402-4414.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B.M., Schulz, R.A., and Olson, E.N. 1995. Requirement of MADS Domain Transcription Factor D-MEF2 for Muscle Formation in Drosophila. *Science (Washington D C)* 267:688-693. 10.1126/science.7839146

Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-DELTADELTACT method. *Methods (Orlando)* 25:402-408. 10.1006/meth.2001.1262

Luo, W., Wu, H., Ye, Y., Li, Z., Hao, S., Kong, L., Zheng, X., Lin, S., Nie, Q., and Zhang, X. 2014. The transient expression of miR-203 and its inhibiting effects on skeletal muscle cell proliferation and differentiation. *Cell Death & Disease* 5. 10.1038/cddis.2014.289

Molkentin, J.D., Black, B.L., Martin, J.F., and Olson, E.N. 1995. Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *CELL* 83:1125-1136. 10.1016/0092-8674(95)90139-6

Molkentin, J.D., Black, B.L., Martin, J.F., and Olson, E.N. 1996. Mutational analysis of the DNA binding, dimerization, and transcriptional activation domains of MEF2C.

355 *MOLECULAR AND CELLULAR BIOLOGY* 16:2627-2636.
356 Nagar, S., Trudler, D., McKercher, S.R., Pina-Crespo, J., Nakanishi, N., Okamoto, S.I., and
357 Lipton, S.A. 2017. Molecular Pathway to Protection From Age-Dependent Photoreceptor
358 Degeneration in Mef2 Deficiency. *Invest Ophthalmol Vis Sci* 58:3741-3749. 10.1167/iovs.17-
359 21767
360 Nebbioso, A., Manzo, F., Miceli, M., Conte, M., Manente, L., Baldi, A., De Luca, A., Rotili, D.,
361 Valente, S., Mai, A., Usiello, A., Gronemeyer, H., and Altucci, L. 2009. Selective class II
362 HDAC inhibitors impair myogenesis by modulating the stability and activity of HDAC-
363 MEF2 complexes. *EMBO REPORTS* 10:776-782. 10.1038/embor.2009.88
364 Ogawa, M., Sakakibara, Y., and Kamemura, K. 2013. Requirement of decreased O-GlcNAc
365 glycosylation of Mef2D for its recruitment to the myogenin promoter. *Biochem Biophys Res*
366 *Commun* 433:558-562. 10.1016/j.bbrc.2013.03.033
367 Ornatsky, O.I., and McDermott, J.C. 1996. MEF2 protein expression, DNA binding specificity
368 and complex composition, and transcriptional activity in muscle and non-muscle cells.
369 *JOURNAL OF BIOLOGICAL CHEMISTRY* 271:24927-24933.
370 Ouyang, H., Chen, X., Li, W., Li, Z., Nie, Q., and Zhang, X. 2018. Circular RNA circSVIL
371 Promotes Myoblast Proliferation and Differentiation by Sponging miR-203 in Chicken.
372 *Frontiers in Genetics* 9:172. 10.3389/fgene.2018.00172
373 Potthoff, M.J., and Olson, E.N. 2007. MEF2: a central regulator of diverse developmental
374 programs. *DEVELOPMENT* 134:4131-4140. 10.1242/dev.008367
375 Runfola, V., Sebastian, S., Dilworth, F.J., and Gabellini, D. 2015. Rbfox proteins regulate
376 tissue-specific alternative splicing of Mef2D required for muscle differentiation. *JOURNAL*
377 *OF CELL SCIENCE* 128:631-637. 10.1242/jcs.161059
378 Sebastian, S., Faralli, H., Yao, Z., Rakopoulos, P., Palii, C., Cao, Y., Singh, K., Liu, Q., Chu, A.,
379 Aziz, A., Brand, M., Tapscott, S.J., and Dilworth, F.J. 2013. Tissue-specific splicing of a
380 ubiquitously expressed transcription factor is essential for muscle differentiation. *GENES &*

DEVELOPMENT 27:1247-1259. 10.1101/gad.215400.113

Singh, R.K., Xia, Z., Bland, C.S., Kalsotra, A., Scavuzzo, M.A., Curk, T., Ule, J., Li, W., and Cooper, T.A. 2014. Rbfox2-coordinated alternative splicing of Mef2d and Rock2 controls myoblast fusion during myogenesis. *MOLECULAR CELL* 55:592-603. 10.1016/j.molcel.2014.06.035

Wang, Y., Wang, J., Liu, H., Zhang, R., Zhang, T., Gan, X., Huang, H., Chen, D., Li, L. 2016. Discovery, Characterization, and Functional Study of a Novel MEF2D CAG Repeat in Duck (Anas platyrhynchos). *DNA and Cell Biology* 35:398-409. 10.1089/dna.2016.3222.

Figure Legends

Figure 1. Analysis of MEF2D protein sequence. (A) The highly conserved functional region of the MEF2D protein sequence. (B) Clustering analysis of MEF2D protein sequences in ten different species. (C) Homology analysis of MEF2D protein sequences in ten different species.

Figure 2. Gene structures of various transcripts of chicken MEF2D. (A) PCR amplification results of MEF2D gene cloning. (B) Gene structures of four novel transcripts. UTR: un-tranlated region; CDS: coding DNA sequence; In/Del: Insertion/deletion.

Figure 3. The expression pattern of different MEF2D variants in various tissues of chicken. Cer, cerebrum; Ceb, cerebellum; Hyp, hypothalamus; Pit, pituitary; Hea, heart; Liv, liver; Spl, spleen; Lun, lung; Kid, kidney; Brm, breast muscle; Lem, leg muscle; Abf, abdominal fat; Suf, subcutaneous fat; Mus, muscular stomach; Gls, glandular stomach.

Figure 4. Expression patterns of MEF2D-V4 in leg muscle at different stage. Different uppercase letters on the error bar indicated extremely significant differences ($P < 0.01$), different lowercase letters indicated significant differences ($P < 0.05$), while the same letters show no significant differences ($P > 0.05$).

Figure 5. Overexpression of MEF2D and RBFOX2 in chicken myoblast. OV-MEF2D-V4 indicates overexpression vector of MEF2D-V4, OV-RBFOX2 indicates overexpression vector of RBFOX2, EGFP control indicates control vector of pEGFP-C1. $^{**}P < 0.01$.

Figure 6. Chicken MEF2D promotes primary myoblast differentiation. (A) The expression of MYOG and MHC was determined by qPCR in primary myoblast after overexpressed MEF2D and RBFOX2. (B) The expression of MYOG and MHC was determined by Western blotting in primary myoblast after overexpressed MEF2D and RBFOX2. OV-MEF2D-V4 or OV-M indicates overexpression vector of MEF2D-V4, OV-RBFOX2 or OV-R indicates overexpression vector of RBFOX2, EGFP control or NC indicates control vector of pEGFP-C1. $^{*}P < 0.05$.

Figure 1

Figure 1. Analysis of MEF2D protein sequence.

(A) The highly conserved functional region of the MEF2D protein sequence. (B) Clustering analysis of MEF2D protein sequences in ten different species. (C) Homology analysis of MEF2D protein sequences in ten different species.

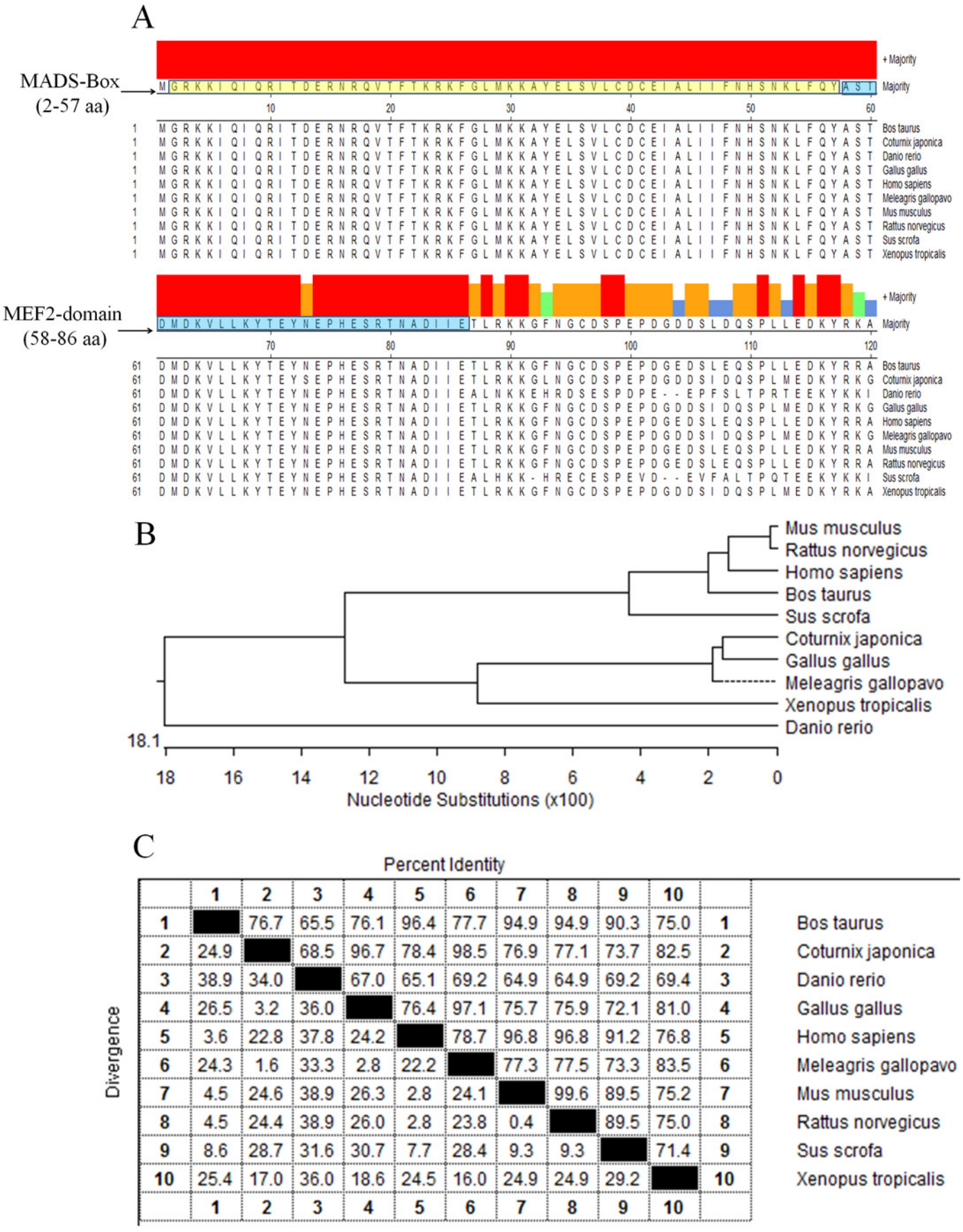
A
MADS-Box
(2-57 aa)
MEF2-domain
(58-86 aa)
+ Majority
Majority
Bos taurus
Coturnix japonica
Danio rerio
Gallus gallus
Homo sapiens
Meleagris gallopavo
Mus musculus
Rattus norvegicus
Sus scrofa
Xenopus tropicalis
B
Mus musculus
Rattus norvegicus
Homo sapiens
Bos taurus
Sus scrofa
Coturnix japonica
Gallus gallus
Meleagris gallopavo
Xenopus tropicalis
Danio rerio
18.1
18 16 14 12 10 8 6 4 2 0
Nucleotide Substitutions (x100)
C
Percent Identity
Divergence
1 2 3 4 5 6 7 8 9 10
1 76.7 65.5 76.1 96.4 77.7 94.9 94.9 90.3 75.0
2 24.9 68.5 96.7 78.4 98.5 76.9 77.1 73.7 82.5
3 38.9 34.0 67.0 65.1 69.2 64.9 64.9 69.2 69.4
4 26.5 3.2 36.0 76.4 97.1 75.7 75.9 72.1 81.0
5 3.6 22.8 37.8 24.2 78.7 96.8 96.8 91.2 76.8
6 24.3 1.6 33.3 2.8 22.2 77.3 77.5 73.3 83.5
7 4.5 24.6 38.9 26.3 2.8 24.1 99.6 89.5 75.2
8 4.5 24.4 38.9 26.0 2.8 23.8 0.4 89.5 75.0
9 8.6 28.7 31.6 30.7 7.7 28.4 9.3 9.3 71.4
10 25.4 17.0 36.0 18.6 24.5 16.0 24.9 24.9 29.2
Bos taurus
Coturnix japonica
Danio rerio
Gallus gallus
Homo sapiens
Meleagris gallopavo
Mus musculus
Rattus norvegicus
Sus scrofa
Xenopus tropicalis

Figure 2

Figure 2. Gene structures of various transcripts of chicken MEF2D.

(A) PCR amplification results of MEF2D gene cloning. (B) Gene structures of four novel transcripts. UTR: un-tranlated region; CDS: coding DNA sequence; In/Del: Insertion/deletion.

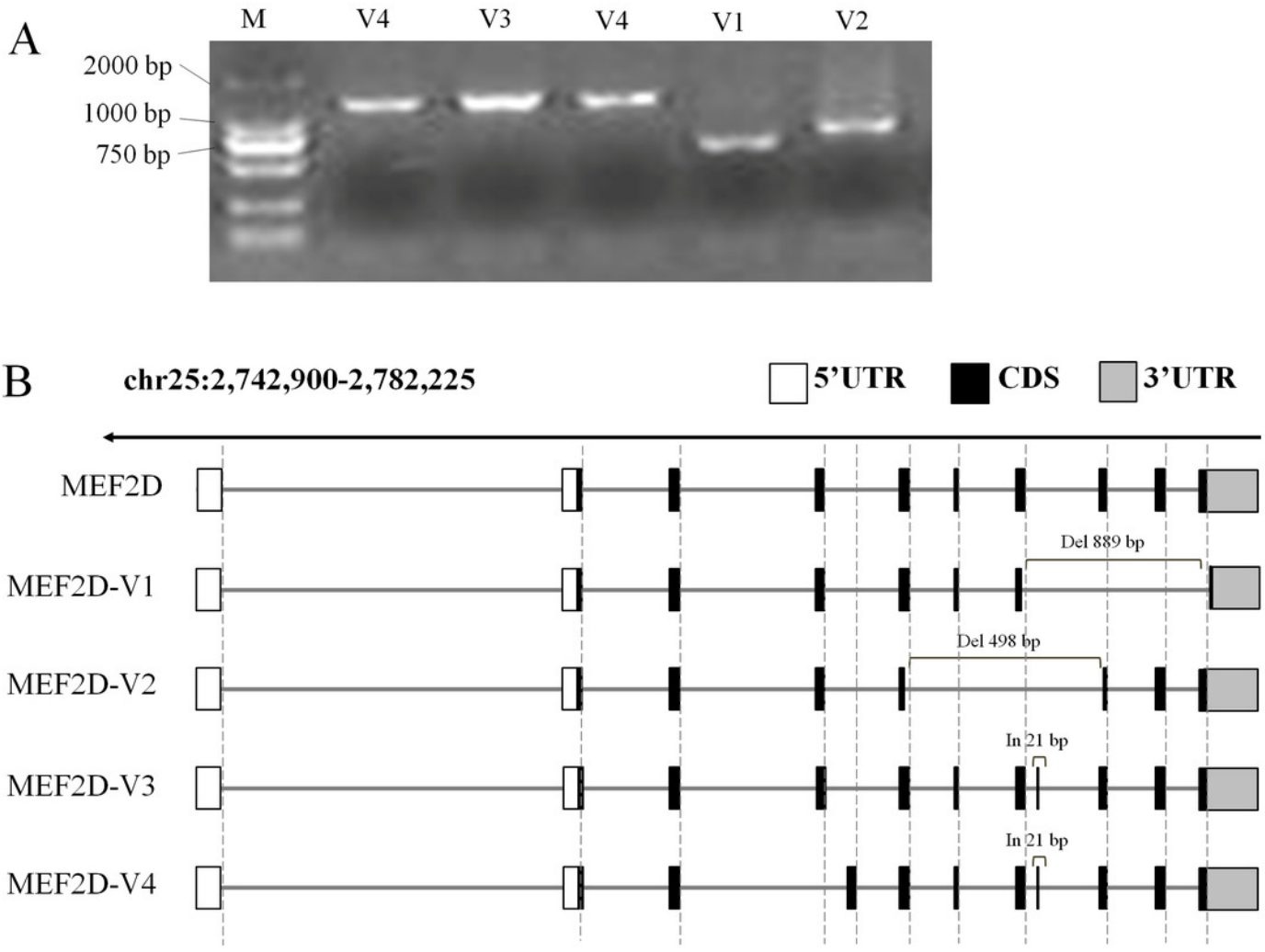

Figure 3

Figure 3. The expression pattern of different MEF2D variants in various tissues of chicken.

Cer, cerebrum; Ceb, cerebellum; Hyp, hypothalamus; Pit, pituitary; Hea, heart; Liv, liver; Spl, spleen; Lun, lung; Kid, kidney; Brm, breast muscle; Lem, leg muscle; Abf, abdominal fat; Suf, subcutaneous fat; Mus, muscular stomach; Gls, glandular stomach.

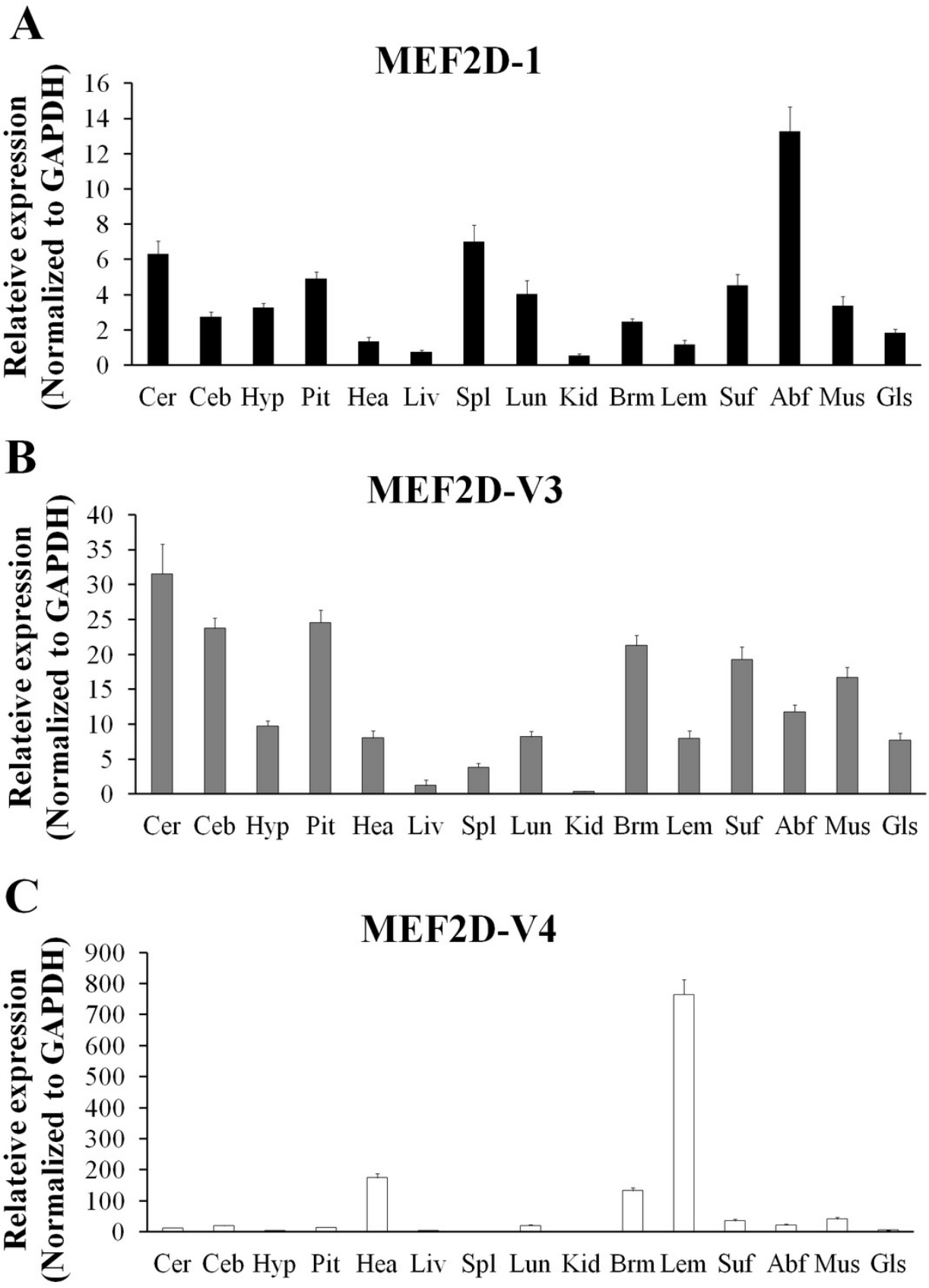
A
MEF2D-1
Relateive expression (Normalized to GAPDH)
16
14
12
10
8
6
4
2
0
Cer Ceb Hyp Pit Hea Liv Spl Lun Kid Brm Lem Suf Abf Mus Gls
B
MEF2D-V3
Relateive expression (Normalized to GAPDH)
40
35
30
25
20
15
10
5
0
Cer Ceb Hyp Pit Hea Liv Spl Lun Kid Brm Lem Suf Abf Mus Gls
C
MEF2D-V4
Relateive expression (Normalized to GAPDH)
900
800
700
600
500
400
300
200
100
0
Cer Ceb Hyp Pit Hea Liv Spl Lun Kid Brm Lem Suf Abf Mus Gls

Figure 4

Figure 4. Expression patterns of MEF2D-V4 in leg muscle at different stage.

Different uppercase letters on the error bar indicated extremely significant differences ($P < 0.01$), different lowercase letters indicated significant differences ($P < 0.05$), while the same letters show no significant differences ($P > 0.05$).

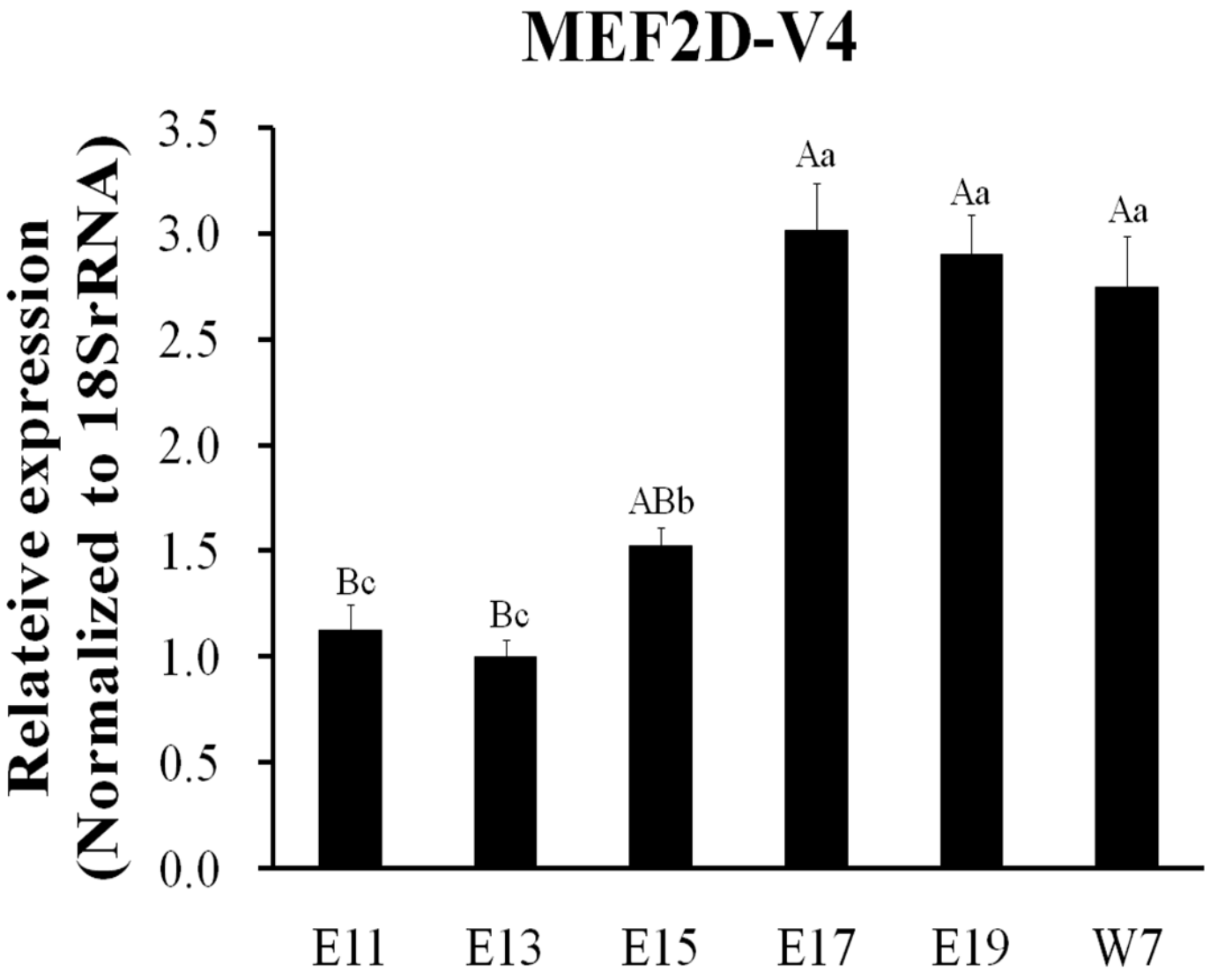

Figure 5

Figure 5. Overexpression of MEF2D and RBFOX2 in chicken myoblast.

OV-MEF2D-V4 indicates overexpression vector of MEF2D-V4, OV-RBFOX2 indicates overexpression vector of RBFOX2, EGFP control indicates control vector of pEGFP-C1. ***P* < 0.01.

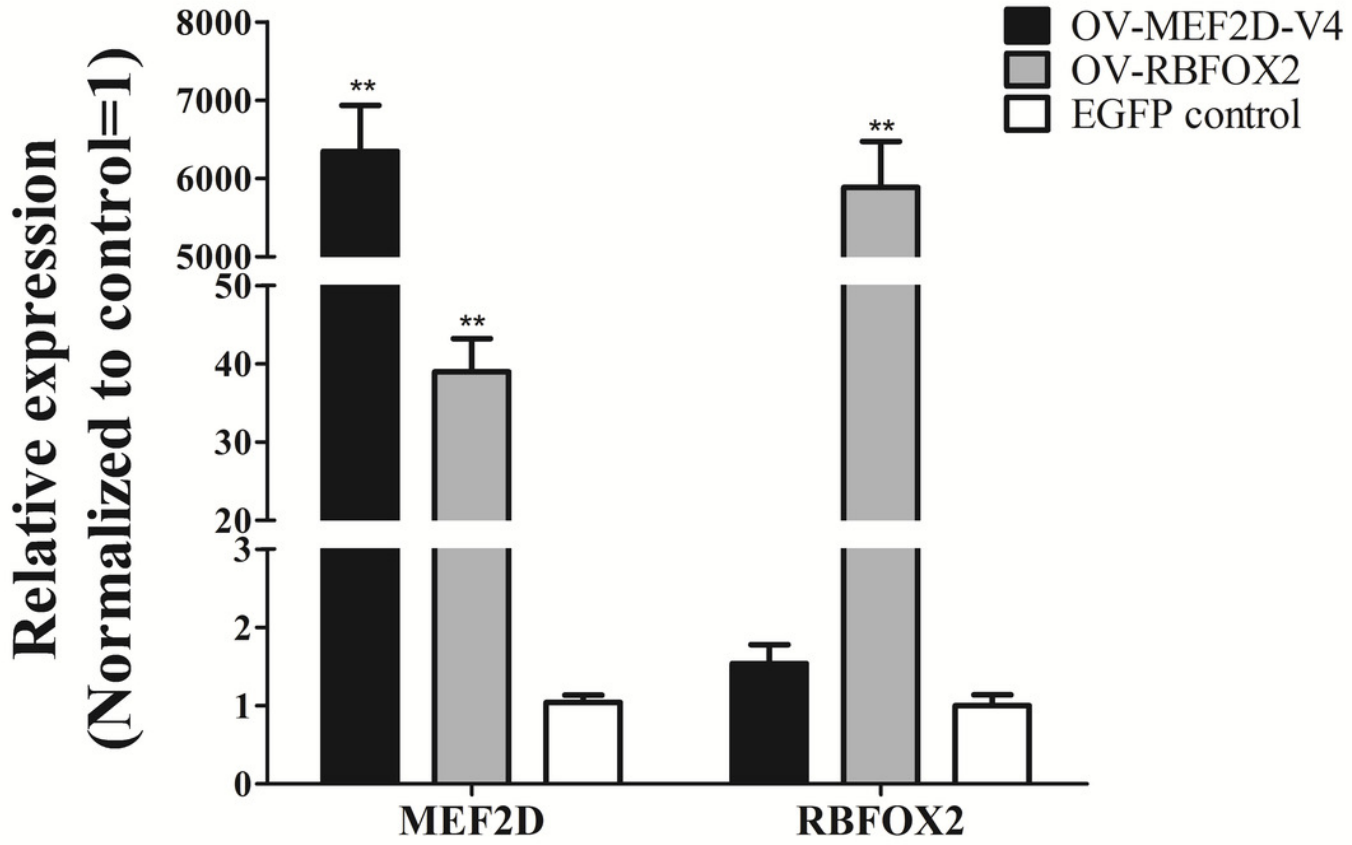

Figure 6

Figure 6. Chicken MEF2D promotes primary myoblast differentiation.

(A) The expression of MYOG and MHC was determined by qPCR in primary myoblast after overexpressed MEF2D and RBFOX2. (B) The expression of MYOG and MHC was determined by Western blotting in primary myoblast after overexpressed MEF2D and RBFOX2. OV-MEF2D-V4 or OV-M indicates overexpression vector of MEF2D-V4, OV-RBFOX2 or OV-R indicates overexpression vector of RBFOX2, EGFP control or NC indicates control vector of pEGFP-C1. *P < 0.05.

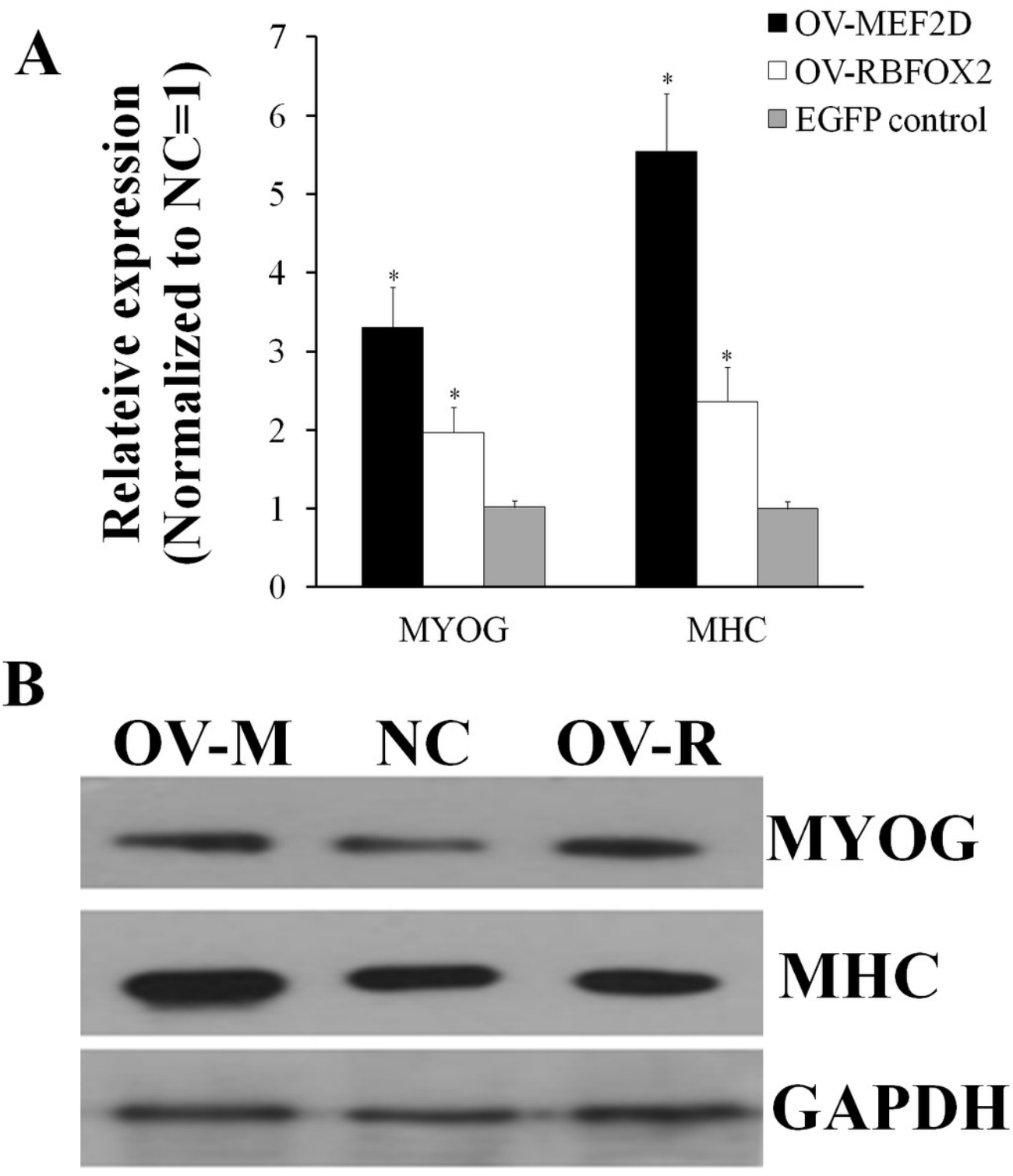
A
Relateive expression
(Normalized to NC=1)
OV-MEF2D
OV-RBFOX2
EGFP control
0
1
2
3
4
5
6
7
*
MYOG
MHC
B
OV-M
NC
OV-R
MYOG
MHC
GAPDH

Table 1(on next page)

Table 1. SNPs in the chicken MEF2D gene.

Table 1. SNPs in the chicken MEF2D gene.

SNP name	Site in the gene	Mutation type	Note
g.26427T > G	exon5	missense	Thr/Pro
g.26446G > T	exon5	missense	Pro/Asn
g.26501G > A	exon5	synonymous	
g.26561T > G	exon5	synonymous	
g.26564C > G	exon5	missense	Gln/His
g.26590A > C	exon5	missense	Val/Gly
g.26608T > G	exon5	missense	Gln/Pro
g.26621A > C	exon5	missense	Ser/Arg
g.28390C > T	exon6	synonymous	
g.28405G > A	exon6	synonymous	
g.28423T > C	exon6	synonymous	
g.30792A > G	exon7	missense	Ser/Pro
g.30808G > A	exon7	missense	Pro/Leu
g.30852A > T	exon7	missense	Ser/Thr
g.30857A > G	exon7	synonymous	
g.30860C > G	exon7	synonymous	
g.30866T > G	exon7	synonymous	
g.30888A > G	exon7	missense	Ser/Pro
g.30892T > G	exon7	missense	Asn/Thr
g.30921T > G	exon7	missense	Thr/Pro
g.33959C > G	exon8	missense	Ala/Pro
g.36092A > G	exon9	synonymous	
g.36094CAGIns/Del	exon9	Insert/Delete	
g.36137A > G	exon9	synonymous	
g.36176A > G	exon9	synonymous	
g.36179A > G	exon9	synonymous	
g.36186C > T	exon9	missense	Gln/stop
g.37162T > C	exon10	synonymous	
g.37187T > G	exon10	missense	Thr/Pro

g.37270T > G	exon10	synonymous	
g.37287T > G	exon10	missense	His/Pro

2

Table 2(on next page)

Table 2. SNP g.36186C > T associated with growth traits in chicken

Table 2. SNP g.36186C > T associated with growth traits in chicken

Traits	*P*-value	Least-mean-squares ± s.e.m		
BW1 (g)	0.0001	26.59 ± 0.64^{C} (TT, 15)	28.84 ± 5.05^{B} (TC, 56)	29.91 ± 0.16^{A} (CC, 251)
BW7 (g)	0.0004	54.90 ± 2.48^{AB} (TT, 15)	55.08 ± 1.17^{B} (TC, 56)	59.85 ± 0.54^{A} (CC, 251)
BW14 (g)	0.0001	116.62 ± 4.61^{AB} (TT, 15)	113.80 ± 2.39^{B} (TC, 56)	125.44 ± 1.13^{A} (CC, 251)
BW21 (g)	0.0007	193.15 ± 8.25^{ABb} (TT, 15)	195.66 ± 4.35^{Bb} (TC, 56)	221.01 ± 2.05^{Aa} (CC, 251)
BW28 (g)	0.0064	294.51 ± 11.95^{AB} (TT, 15)	291.62 ± 6.19^{B} (TC, 56)	312.22 ± 2.94^{A} (CC, 251)
BW63 (g)	0.0264	949.07 ± 40.02^{ab} (TT, 15)	965.01 ± 23.14^{b} (TC, 56)	1023.99 ± 11.65^{a} (CC,251)
SL42 (mm)	0.0159	59.38 ± 1.07^{AB} (TT, 15)	59.20 ± 0.58^{B} (TC, 52)	60.90 ± 0.26^{A} (CC, 248)
SL77(mm)	0.0417	84.23 ± 1.67^{b} (TT, 9)	88.34 ± 1.06^{a} (TC, 23)	88.80 ± 0.60^{a} (CC, 69)
SL84(mm)	0.0465	86.95 ± 1.81^{ab} (TT, 9)	90.96 ± 0.95^{a} (TC, 33)	88.63 ± 0.43^{b} (CC,157)
SD42 (mm)	0.0069	7.75 ± 0.17^{AB} (TT, 15)	7.56 ± 0.093^{B} (TC, 52)	7.89 ± 0.043^{A} (CC, 248)
SD56 (mm)	0.014	8.63 ± 0.20^{AB} (TT, 15)	8.47 ± 0.10^{B} (TC, 56)	8.79 ± 0.049^{A} (CC, 248)
0-4Wks ADG(g/w)	0.0149	9.57 ± 0.42^{AB} (TT, 15)	9.38 ± 0.22^{B} (TC, 56)	10.07 ± 0.10^{A} (CC, 251)

Note: BW, body weight; SL, shank length; SD, shank diameter; 0-4WKs ADG (g/w), 0-4 weeks of average weight gain (g/week). Letters and numbers in bracket refer to genotype and number of chickens

with that genotype. $^{a,b}P < 0.05$; $^{A,B}P < 0.01$.

Table 3(on next page)

Table 3. SNP g.36094CAGIns/Del associated with carcass traits in chicken

Table 3. SNP g.36094CAGIns/Del associated with carcass traits in chicken

Traits	*P*-value	Least-mean-squares ± s.e.m		
EW (g)	0.0042	1093.2 ± 107.3^{A}	1075.5 ± 114.2^{AB}	1024.9 ± 102.5^{B}
		(Ins/ins, 62)	(Ins/del, 80)	(Del/del, 160)
LMW (g)	0.013	119.5 ± 13.2^{a}	117.4 ± 14.3^{a}	112.6 ± 10.8^{b}
		(Ins/ins, 62)	(Ins/del, 80)	(Del/del, 160)
AFW(g)	0.021	24.33 ± 2.54^{b}	28.92 ± 4.24^{a}	26.91 ± 4.16^{ab}
		(Ins/ins, 54)	(Ins/del, 69)	(Del/del, 142)
SIL (mm)	0.028	133.6 ± 15.4^{ab}	149.5 ± 11.5^{a}	136.2 ± 10.2^{b}
		(Ins/ins, 50)	(Ins/del, 62)	(Del/del, 137)

Note: EW, eviscerated weight; LMW, leg muscle weight; AFW, abdominal fat pad weight; SIL, small intestine length. Letters and numbers in bracket refer to genotype and number of chickens with that genotype. $^{a,b}P < 0.05$; $^{A,B}P < 0.01$.