Research Article

Molecular cloning and *in silico* analysis of the duck (*Anas platyrhynchos*) MEF2A gene cDNA and its expression profile in muscle tissues during fetal development

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Abstract

The role of myogenic enhancer transcription factor 2a (MEF2A) in avian muscle during fetal development is unknown. In this work, we cloned the duck MEF2A cDNA sequence (GenBank accession no. HM460752) and examined its developmental expression profiles in cardiac muscle, non-vascular smooth muscle and skeletal muscle. Duck MEF2A cDNA comprised 1479 bp encoding 492 amino acid residues. *In silico* analysis showed that MEF2A contained MADS (MCM1, AGAMOUS, DEFICIENS and SRF - serum response factor), MEF2 and mitogen-activated protein kinase (MAPK) transcription domains with high homology to related proteins in other species. Modified sites in these domains were conserved among species and several variants were found. Quantitative PCR showed that MEF2A was expressed in all three muscles at each developmental stage examined, with the expression in smooth muscle being higher than in the other muscles. These results indicate that the conserved domains of duck MEF2A, including the MADS and MEF2 domains, are important for MEF2A transcription factor function. The expression of MEF2A in duck smooth muscle and cardiac muscle suggests that MEF2A plays a role in these two tissues.

Key words: duck MEF2A, expression profile, *in silico* analysis, molecular cloning, muscle tissues.

Introduction

Animals possess three major types of muscle, namely, cardiac, skeletal and smooth muscle that are essential for movement (Byers *et al.*, 1991; Handschin *et al.*, 2007; Leong *et al.*, 2008; MacDonald, 2008). The developmental stages from embryo formation to birth are important for muscle development (Rehfeldt, 2005). The skeletal muscle mass of adult animals is determined early in development and, in most animals, there is no post-natal increase in the number of myofibers. Post-natal muscle hypertrophy involves only myofiber lengthening and thickening (Chauvigne, 2005). Although the heart is one of the earliest organs formed during embryogenesis (Forouhar *et al.*, 2006), subsequent development is nevertheless important because heart muscle development is completed during this stage (Noorlander *et al.*, 2008). The fetal stage is also important for smooth muscle maturation and the initiation of physiological functions (Olson, 2006). Muscle development in this stage is regulated by many genes such as muscle regular factors (MRFs) and myostatin (MSTN) (Owens *et al.*,

2004; Olson, 2006; Potthoff and Olson, 2007). However, only the myogenic enhancer transcription factor 2a (MEF2A) gene has been reported to be expressed in all three muscle tissues (Edmondson *et al.*, 1994; Wang *et al.*, 2000).

MEF2A is a muscle-specific factor that belongs to the MEF2 gene family. In mammals, four members of the gene family have been identified, *i.e.*, MEF2A, -B, -C and -D. MEF2A is involved in a variety of muscle responses, including skeletal muscle myofiber differentiation, smooth muscle maturation, and normal development of cardiac muscle (Edmondson *et al.*,, 1994; Wang *et al.*,, 2000; Potthoff and Olson, 2007). In addition to muscle, MEF2A is expressed in neuronal cells and at low levels in a wide range of cell types during embryogenesis and adulthood. In skeletal muscle, MEF2A plays a cooperative role with the MRF gene family in inducing muscle hypertrophy (Molkentin *et al.*, 1995; Black and Olson, 1998), and its polymorphisms are associated with growth traits in Chinese indigenous cattle breeds (Chen *et al.*, 2010) and with muscle growth in chickens (Zhou *et al.*, 2010). MEF2A is also highly expressed in the heart during zebrafish embryogenesis (Wang YX *et al.*, 2005). Mice lacking MEF2A display an array of cardiovascular defects that cause most to

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die suddenly (Naya *et al.*, 2002). MEF2A polymorphisms have been associated with several cardiac diseases in human and may account for 1.93% of such diseases (Wang Q *et al.*, 2005). The MEF2A gene also plays a role in smooth muscle cell proliferation and contraction and is expressed in smooth muscle mainly during adulthood (Firulli *et al.*, 1996). Almost all of the studies of MEF2A have focused on in its functions during embryogenesis and in adulthood. In contrast, there have been no studies of the roles of MEF2A in muscle tissues during fetal development.

Most studies of MEF2A have used mammals as a model, although MEF2A and MEF2D have also been studied in birds (Buchberger and Arnold, 1999; Xue *et al.*, 2000). Buchberger and Arnold (1999) reported that chicken MEF2A has a role avian in skeletal muscle and cardiac muscle, although the precise functions of this protein are still unclear. In this work, we examined the functions of MEF2A in muscle tissue from the Pekin duck, a famous meat poultry breed. The coding sequence of duck MEF2A was cloned and its expression profile in muscle during fetal development was determined using real-time PCR. These results provide essential data for elucidating the roles of MEF2A in muscle tissue and may also provide new clues for improving muscle production in duck breeding.

Materials and Methods

Tissues collection and total RNA extraction

All procedures were approved by the Guidelines on Humane Treatment of Laboratory Animals (2006). Twenty five Pekin duck (*Anas platyrhynchos*) embryos (embryonic age: 10, 14, 18, 22 or 27 days old, designated E10d, E14d, E18d, E22d and E27d, respectively) and five neonates (one week post-natal, designated as p7d) were used. All of the embryos and birds were from the Sichuan Agricultural University Waterfowl Breeding Experimental Farm. The hatching embryos and birds were maintained under the same conditions. For each embryo and newborn bird, approximately 2 g of tissue was collected from the heart, small intestine and leg muscle. The samples were immediately frozen in liquid nitrogen and stored at -86 °C for RNA extraction. Total RNA was extracted using Trizol (Invitrogen, USA) according to the manufacturer's instructions

and then measured spectrophotometrically. First-strand cDNA was obtained from 10 µg of total RNA using a cDNA synthesis kit (Takara, Japan) according to the manufacturer's instruction.

Cloning and sequencing of the duck MEF2A CDS

All of the primers were designed using Primer Premier 5 software and were synthesized by the Shanghai Yingjun Biology Company. The primers used to amplify the duck MEF2A gene coding sequence (CDS) are shown in Table 1. Two microliters of the cDNA mix was used as a template in a 20 µL PCR reaction volume. The reaction conditions included denaturation for 5 min at 94 °C followed by 30 cycles of amplification at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis on a 1% agarose gel and purified using a gel extraction kit (Watson Biomedical Inc., Shanghai, China). The purified PCR products were ligated into the pGEM-19T vector (Invitrogen). Positive clones were selected and sequenced by the Shanghai Yingjun Biology Company. The cDNA segments obtained from sequencing were edited and assembled with Editseq and Seqman in DNAstar software (DNASTAR Inc., Madison, WI, USA)

Real-time PCR

The levels of MEF2A mRNA expression in duck muscles at different developmental stages were measured by real-time PCR using a 96-well iCycler IQ5 (BioRad, USA) and Takara ExTaq RT-PCR kits with SYBR green (Takara, Dalian, China). One sample was collected from each muscle type, each bird and each stage, and the real-time PCR were repeated three times together for each sample. Duck β-actin (EF667345) and duck glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, AY436595), the sequences of which were obtained from GenBank, were used as internal controls. The real-time PCR primers were designed using Primer 5 software (Primer Biosoft International, USA) and are shown in Table 2. The primers were synthesized by the Shanghai Yingjun Biology Company. Amplicons corresponding to each target gene were examined by agarose gel electrophoresis to confirm the presence of a single band of the expected size. The procedure for

Table 1 - PCR primers used for cloning of MEF2A coding sequence.

MEF2A	Primer sequence	Product length (bp)	Annealing temperature (°C)	Reference gene information
Primer 1	F: 5'ATGGGGAGAAAGAAAATACA3' R: 5'AAGTAATCGTCAGCATCAGG3'	314	55	Chicken (NM_204184)
Primer 2	F: 5' GAAGAGGAAGTTTGGATT3' R: 5' ATGTTGATGTTTTGGTTG3'	1143	51	Human (NM_002479)
Primer 3	F: 5' CAGGGACTGGTGTATTCGG3' R: 5' TGTCACCCACGTGTCCATC3'	528	52.5	Mouse (NM_001033713)

F, R – forward and reverse primers, respectively.

Table 2 - Primers for real-time PCR analysis.

Gene	Primer sequence	Product length (bp)	Tm (°C)	Amplification efficiency (%)
MEF2A	F: 5' GGGTATGATGCCACCATTGAA 3' R: 5' GGTCTGCGCTAGTCAAGGAGTAA 3'	170	59.5	98
β-actin	F: 5' GCTATGTCGCCCTGGATTTC 3' R: 5' CACAGGACTCCATACCCAAGAA 3'	168	59.6	97
GAPDH	F: 5' AAGGCTGAGAATGGGAAAC 3' R: 5' TTCAGGGACTTGTCATACTTC 3'	254	53.9	95

F, R – forward and reverse primers, respectively.

real-time PCR was as follows: 10 s of pre-denaturation at 95 °C, followed by 45 cycles of 95 °C for 5 s and 62 °C for 30 s. PCR products were diluted 16-fold and used to generate the calibration curve and determine the PCR amplification efficiency (E*ff*) for each gene (MEF2A, β-actin and GAPDH, provided in Table 2).

The relative mRNA expression for target genes was determined with the following formula after obtaining the C_t value of the gene of interest (MEF2A) and the C_t value of the internal control genes:

$$\text{Rel. quantity} = \frac{\dfrac{\text{GOI}_{\text{sample}}}{\text{GOI}_{\text{control}}}}{\dfrac{\text{Norm}_{\text{sample}}}{\text{Norm}_{\text{control}}}} = \frac{(1+\text{E}ff)_{\text{GOI}}^{(\text{Ct}_{\text{control}}-\text{Ct}_{\text{sample}})}}{(1+\text{E}ff)_{\text{Norm}}^{(\text{Ct}_{\text{control}}-\text{Ct}_{\text{sample}})}}$$

The relative quantity (Rel. quantity) is the multiple of the differential expression of the gene of interest (GOI) between the test samples and control samples. The normalizer (Norm) is the reference gene and the PCR amplification efficiency is represented by E*ff*.

Statistical analysis

BLASTn (NCBI) was used to identify the gene. Analysis of the nucleotide and amino acid sequences, prediction of secondary structure and multiple sequence alignment were done with Editseq, Protean, and MegAlign in DNAstar, respectively. Conserved domains in the protein were analyzed using the CD-search tool available from NCBI. Phylogenetic trees were constructed with MEGA 4.0 software by the neighbor-joining method and bootstrap sampling 1,000 times.

The real-time PCR data were subjected to analysis of variance (ANOVA) and the means were compared for significance by Tukey's test. The ANOVA and *t*-tests were done with SAS (SAS Institute, Cary, NC, USA) software. A *p*-value of $p < 0.05$ was considered statistically significant.

Results

Comparison of duck MEF2A with other species

After sequencing and assembling of the fragments, the total coding sequence of duck MEF2A was obtained and submitted to GenBank (GenBank accession no.

HM460752). This consisted of 1479 nucleotides encoding 492 amino acids and showed high similarity with the chicken sequence (92.8% similarity at the nucleotide level and 93.5% at the amino acid level; Table 3).

Phylogenetic tree construction

The phylogenetic tree constructed based on vertebrate MEF2A amino acid sequences showed that the sequences formed three main groups: mammalian MEF2A, fish MEF2A and avian MEF2A (Figure 1). A CD-search on NCBI revealed that duck MEF2A had three conserved domains in the peptide strands, *i.e.*, the MADS (aa 3-57), MEF2 (aa 58-86) and MAPK (mitogen-activated protein kinase) binding domains (aa 264-285), but lacked a beta do-
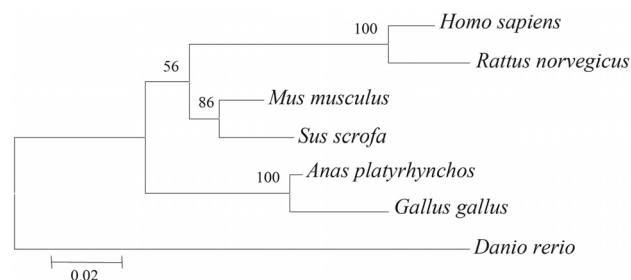
Figure 1 - Phylogram of MEF2A amino acid sequences constructed using the neighbor-joining method. The predicted amino acid sequence of duck MEF2A and the long form MEF2A of other species were used in this analysis. The phylogenetic tree was constructed using MEGA software with the neighbor-joining method and bootstrap resampling (1,000 times). The numbers indicate the bootstrap value (%). Reference species: *Gallus gallus* (chicken) NP_990195, *Homo sapiens* (human) NP_005578, *Bos taurus* (ox) NP_001077107, *Sus scrofa* (pig) EF576923, *Mus musculus* (mouse) ABQ53160 and *Danio rerio* (zebrafish) NP_571376.

Table 3 - Homology of MEF2A in several vertebrate species.

Species [*Latin name*]	Nucleotide (%)	Amino acid (%)
Chicken [*Gallus gallus*]	92.8	93.5
Human [*Homo sapiens*]	80.4	84.2
Mouse [*Mus musculus*]	82.2	88.2
Ox [*Bos taurus*]	80.4	82.8
Pig [*Sus scrofa*]	82.7	86.0
Zebrafish [*Danio rerio*]	74.1	73.8
African frog [*Xenopus laevis*]	71.3	75.7

main that is present in other organisms, except for zebrafish. There were no amino acid variants in the three conserved domains of the duck and chicken proteins. These three regions were also highly conserved between birds and mammals. Only two variant residues were observed in the porcine MEF2A, MADS and MEF2 domains compared with other mammals, in addition to a single residue change from 'N' in avian sequences to 'S' in mammalian and fish (zebrafish) sequences; the latter also showed two additional amino acid changes compared to the avian sequences.

Several modified residues were identified in MEF2A in addition to the conserved domains of MEF2A. Most of these were conserved among species, including acetyllysine sites at positions 117, 248, 253 and 394, phosphoserine sites at positions 98, 221, 233, 346, 399 and 439, and phosphothreonine sites at positions 302 and 310. Several

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                    MADS domain                                    MEF2 domain
Duck       MGRKKIQITRIMDERNRQVTFTKRKFGLMKKAYELSVLCDCEIALIIFNSSNKLFQYASTDMDKVLLKYTEYNEPHESRT    80
Chicken    --------------------------------------------------------------------------------    80
Human      --------------------------------------------------------------------------------    80
Mouse      --------------------------------------------------------------------------------    80
Swine      ------------------------------------------------------t----------i--------------    80
Zebrafish  --------------------------------------------------------------------------------    80
                                                                       D1 Region
Duck       NSDIVETLRKKGLNGCESPDADDYFEHSPLSEDRFSKLNEDSDFIFK..RGPPGLPAQNFSMSVTVPVSNPNTLTYSNPG   158
Chicken    ------------------------------------------------..----------------------------   158
Human      -----a-n--ehr--d---p-tsyvlt-ht-ekyk-i--ef-nmmrnhkia----p------------ts--a-s-t---   160
Mouse      ----------------------------------------..-----p------------ts--a-s-t---   158
Swine      ------------------l---------------------..-----p------------ts--a-s-t---   158
Zebrafish  -----k--n--h-d-p---p--c-g----md---g----e--lmy-r.c--ta--p----h-a---t---ams-.--   158
                D2 Region
Duck       SSLVSPSL..AASSSLTDTTMLSPPQTTLHRNVSPGAPQRPPSTGNAGAMLGTTDLTVPNGAGTCPVGNGFVNSRASPSL   236
Chicken    --------..-------s-----------------------------gi---------s-----vw--------   236
Human      --------..----t--ss---------------------------g--s---------ss----------n-   238
Mouse      --------..----t-a-ss-----pa-------------------s--sg--s---------ns------n-   236
Swine      --------..----t-a-ss-----a--------------------s--g--s-s---------ss---...........   224
Zebrafish  a--s-q--sa--aa--s-ga-----gsm--s-v-..--------st--..n-fvnprgsp--llgt-s---.........   224
                                                                       Beta domain
Duck       LGTTGGGNGLGKVMPTKSPPPPGGGNLGMNNRKPDLRVVIPPSSKGMMPPLN........TQRISSSQSTQPLATPVVSV   308
Chicken    ---a--------------------g--------------------teedeleln-------------------   316
Human      i-a--a.-s---------------s---------seeeeleln-------a----------   317
Mouse      i-n--a.-s---------s---s---------seeeeleln a-------a----------   315
Swine      ....................-----s---------seeeeleln-------ap---------   281
Zebrafish  ........-----a-----.--m-igs----------.......-   286
                For interaction with MAPKs     D3 Region
Duck       TTPSLPPQGLVYSAMPTAYNTDYSLTSADLSALQGFNSPGMLSLGQVSAWQQHHLGPATLSSLVTGSQLSQGSNLSINTN   388
Chicken    ----f--a------------------fe---------p---------------s---------------   396
Human      ---------------------------------------q-a-----a-g---------   397
Mouse      --------------------------t---------a-------q-a-----a-g---------   395
Swine      ----q------------------------------------q-a-----a-g---------   361
Zebrafish  ----a------g-----se---s-e--s---g---.--i-s------q--q-a-----g-gh-p---------s   365
                V1 Region
Duck       QNINIKSEPISPPRDRVTPSGFPQQQ...QPQQQQQQ.....QQQQQQQQQSRQEMGRSPVDSLSSSSSSYDGSDREDP   458
Chicken    ----------ns----.....p---p-pp.....-pp--pp-.---------------------   465
Human      ---s--------m----q-------....q-q----ppppppqp-p-pp-p-p--------------   473
Mouse      ------------m----q--.-----.....-qp--pp.....p-p.p-p-p----------------   464
Swine      ------------m----q--pppps-ap-p-ppq....p-p-p-p-a---------------   437
Zebrafish  --v---------e-------p-.........--pp...........sg-pd-----------c---------h   424

Duck       RSDFHSPVVLGRPPN..SEDRESPSVKRMRMDTWVT   492
Chicken    ---------------..-------------------   499
Human      -g-----i-------..t-------------a---   507
Mouse      -g-----i-------..t-----------------   498
Swine      -g-------------..t-------------a---   471
Zebrafish  -p-----lg-----aggade---------------   460
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Figure 2 - Alignment of the predicted amino acid sequence of duck MEF2A with the corresponding sequences of other vertebrates. The numbers on the right indicate the residue positions. Dashes (- - -) indicate the same (unchanged) amino acids and dots () indicate the absence of amino acids (insertion of spaces to improve alignment). The sequences were compared using DNAMAN software. The GenBank accession numbers of each species are as follows: *Gallus gallus* (chicken) NM_204864, *Homo sapiens* (human) NM_005587, *Sus scrofa* (pig) EF576923, *Mus musculus* (mouse) NM_001033713 and *Danio rerio* (zebrafish) NP_571376.1. K - acetyllysine site, S - phosphoserine site and T - phosphothreonine site.

modified sites differed between birds and mammals, such as the phosphoserine sites at positions 148, 153, 175, 176, 208 and 267. The modified sites could be grouped into four regions, identified as regions 1, 2 and 3 (D1, D2, and D3, respectively) and a region of variants 1 (V1) (Figure 3). The D1-D3 regions had the same characteristics as avian (duck and chick) MEF2A, which differed from those of mammals. The V1 region varied in all of the species examined. The secondary structures of the D1-D3 and V1 regions were predicted using the protean program in DANstar, and the results showed that the secondary structure distributions in the D1-D3 regions differed between birds and mammals (Figure 3).

Expression profiles of duck MEF2A in muscles during fetal development

Figures 4-6 show the expression profiles of duck MEF2A. MEF2A was expressed in skeletal, smooth and cardiac muscle at each developmental stage examined. The expression level was higher in smooth muscle than in the other two muscles. In cardiac muscle, MEF2A showed a significant change (increase) only at embryo stage 27d, when the eggs were close to hatching. In skeletal muscle, MEF2A expression increased from embryo stage 10d to 18d, after which it decreased from embryo stage 22d onwards; at postnatal 7d the expression was lower than in any of the preceding periods. In smooth muscle, there was a progressive increase in MEF2A expression up to 22d, followed by a slight decline in later periods.

Discussion

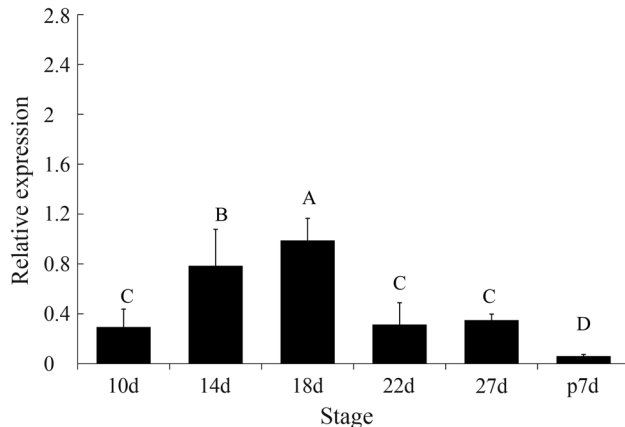### Identification and functional prediction of duck MEF2A

MEF2A is one of the MEF2 gene family members and in birds has so far been cloned only from chicken (Buchberger and Arnold, 1999). Duck MEF2A is the second member of avian origin to be cloned. It shared > 90% identity with the chicken protein at the nucleotide and amino acid levels. An *in silico* analysis showed that duck MEF2A contained a MADS domain in its N-terminal, followed by a MEF2 domain. This structural organization was similar to the corresponding chicken and mammalian proteins (Buchberger and Arnold, 1999; Huang *et al.*, 2000; Wu *et al.*, 2010). The MADS domain is reportedly involved in specific DNA binding and dimerization while the MEF2 domain increases DNA binding and mediates the interaction of MEF2 proteins with other cofactors such as GATA, ERK5 and HDAC. The duck MEF2A gene also had an evolutionarily conserved regulatory region for interaction with MAPKs. The three conserved domains (MADS, MEF2 and MAPK) show few variants among different organisms, which suggests that duck MEF2A plays a similar role to the corresponding protein in other species.
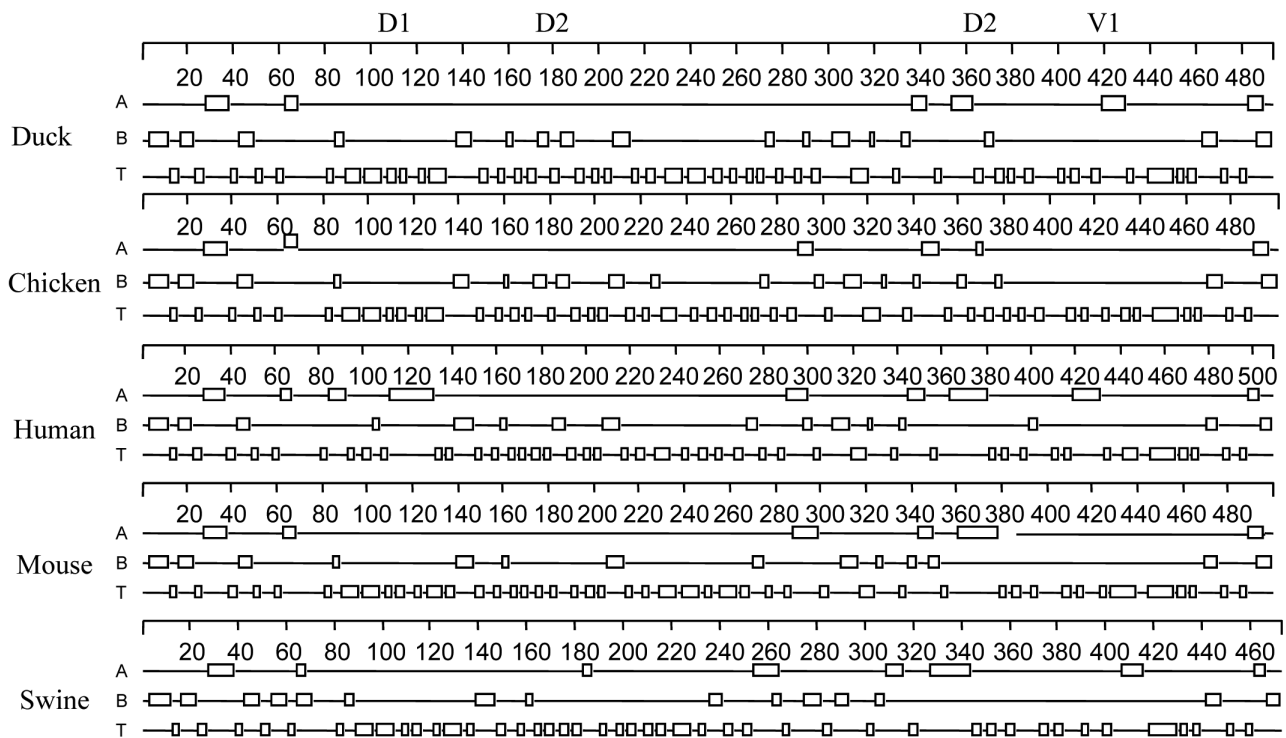
Figure 3 - Secondary structure prediction of the D1, D2, D3 and V1 regions in Figure 1 and prediction of processing using the protean program in DNAstar software, based on the Chou-Fasman mathematic model. The numbers in the figure represent amino acid positions. A - α helix, B - β sheet and T - random turn.
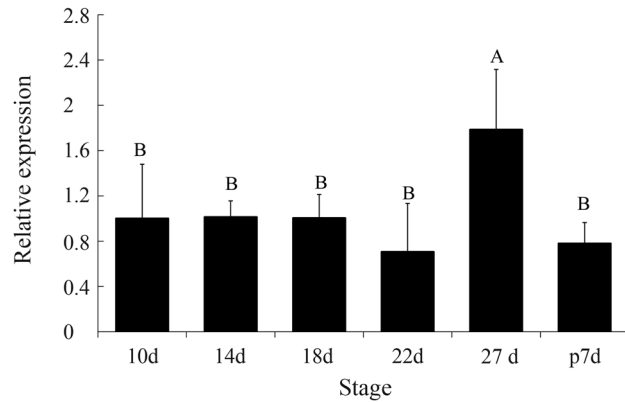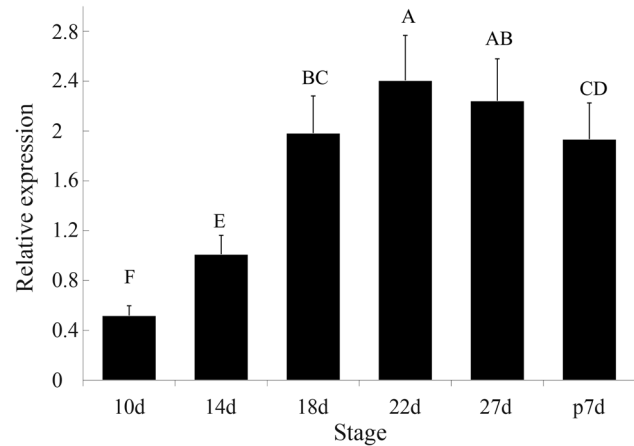
Figure 4 - Expression profile of MEF2A in duck cardiac muscle during development. Different letters indicate a significant difference (p < 0.05) in gene expression during development whereas the same letters indicate no difference. p7d - first post-natal week. The columns represent the means ± SEM of 5 muscles in each stage.

Figure 6 - Expression profile of MEF2A in duck smooth muscle during development. Different letters indicate a significant difference (p < 0.05) in gene expression during development whereas the same letters indicate no difference. p7d - first post-natal week. The columns represent the means ± SEM of 5 muscles in each stage.

Figure 5 - Expression profile of MEF2A in duck skeletal muscle during development. Different letters indicate a significant difference (p < 0.05) in gene expression during development whereas the same letters indicate no difference. p7d - first post-natal week. The columns represent the means ± SEM of 5 muscles in each stage.

Vertebrate MEF2A amino acid sequences contain many modified sites that could be involved in MEF2A function (Zhao *et al.*, 1999). Most of the modified sites in the conserved regions are the same among different organisms, indicating a role in regulating the stability and function of MEF2A (Cox *et al.*, 2003). The MAPK domain in the C-terminal region of MEF2A may play a role in signal transduction (Barsyte-Lovejoy *et al.*, 2004). Figure 2 shows an amino acid substitution in the MAPK domain, *viz.* an Asn residue in birds that is Ser (a phosphorylation site) in mammals. This substitution suggests that the function of the MAPK domain in birds may be different from that in mammals. There are also modified sites outside of the conserved domains, including Ser408 (Ser399 in duck), Ser355 (Ser346 in duck), Lys403 (Lys394 in duck), Thr312 (Thr303 in duck) and Thr319 (Thr310 in duck). Phosphorylation and dephosphorylation of Ser408 in human

MEF2A prevents acetylation of Lys403 and acetylation of Lys403, respectively, which inhibits dendrite claw differentiation in mammals (Cox *et al.*, 2003; Flavell *et al.*, 2006; Shalizi *et al.*, 2006). Phosphorylation of Thr312 and Thr319 is involved in p38 MAPK signaling and the activation of transcription (Zhao *et al.*, 1999; Han and Molkentin, 2000; Lee *et al.*, 2000). Phosphorylation of Ser408 by CDK5, which is induced by neurotoxicity, inhibits MEF2A transcriptional activation, leading to apoptosis of cortical neurons (Gong *et al.*, 2003). Phosphorylation of Thr312, Thr319 and Ser355 can be induced by EGF sumoylation at Lys403, which is enhanced by PIAS1 and represses transcriptional activity (Kato *et al.*, 2000). Acetylation of Lys4 increases DNA binding, transactivation and hyperacetylation by p300, leading to enhanced cardiac myocyte growth and heart failure (Angelelli *et al.*, 2008). These modified sites are conserved among birds and mammals, indicating similarly conserved functions in different organisms.

Developmental expression of MEF2A in duck muscle during the muscle development stage

Previous studies of MEF2A have focused on its functions during embryogenesis and in adulthood. Our results show that duck MEF2A was expressed in cardiac, smooth and skeletal muscle during fetal development. In skeletal muscle, MEF2A expression increased to reach a peak at embryonic day 18, followed by a subsequent decrease. Buchberger and Arnold (1999) reported that in chickens MEF2A expression was not detected in newly formed somites until the muscle-specific transcription factors MyoD and myogenin were present, indicating that activation of the MEF2A gene in skeletal muscle was dependent on these basic helix-loop-helix transcription factors. We have examined the developmental expression of duck MyoG and MRF4 in skeletal muscle during the fetal stage and found

that MyoG and MRF4 are highly expressed in the leg muscles at embryonic days 14d and 18d, respectively (Liu *et al.*, 2010), which is before the peak expression of MEF2A. Our results are consistent with those of Buchberger and Arnold (1999) and suggest that activation of the MEF2A gene in skeletal muscle is dependent on these basic helix-loop-helix transcription factors.

Myocardial muscle develops mainly during early embryogenesis and MEF2A is highly expressed in zebrafish heart during embryogenesis (Mathavan *et al.*, 2005; Wang Q *et al.,* 2005). In chickens, MEF2A expression is first observed in the precardiac mesoderm of HH stage 8 embryos, and expression in heart and skeletal muscle continues into adulthood, when it is also observed in the intestinal mesenchyme and brain. During later embryonic development, MEF2A expression continues in the heart tube and later in the atrium and ventricle (Buchberger and Arnold, 1999; Czubryt and Olson, 2004). Our results support the above findings since MEF2A was found to be expressed at a steady level up to 22d, with a peak at 27d. This change in expression as the egg approaches hatching may enable the duckling to adapt to a new environment when the heart will mature further.

MEF2A is thought to be important for the activation and expression of contractile proteins and other muscle-specific factors in skeletal and cardiac muscle (Czubryt and Olson, 2004). Firulli *et al.* (1996) showed that MEF2A is highly expressed in smooth cells producing contractile proteins. Duck MEF2A was highly expressed in smooth muscle at almost all of the fetal stages, with greater expression than in skeletal and cardiac muscle. The elevated expression of duck MEF2A in smooth muscle during these stages suggests that this protein has an important role in smooth muscle development.

Relationship between MEF2A expression and protein structure

MEF2 binds directly to the promoters or enhancers of the majority of muscle-specific genes (Naya and Olson, 1999), and virtually all muscle genes, including MRFs, have MEF2 binding sites in their regulatory regions (Messina *et al.*, 2010). The MADS box and adjacent MEF2 domain of MEF2 proteins play a cooperative role in binding to the regulatory regions of muscle genes (Potthoff and Olson, 2007; Ramachandran *et al.*, 2008) to activate skeletal muscle differentiation. However, unlike MRFs, MEF2 factors cannot activate myogenesis alone but, instead, combine with MRFs through protein-protein interactions in the MADS box and bHLH domain to vastly improve the efficiency of the myogenic program (Kaushal *et al.*, 1994). The MEF2A domains in poultry are homologous to those of mammals. Duck MEF2A lacks a beta domain region that is present in chickens and other species; this domain may be alternatively spliced in humans (Yu *et al.*, 1992). The RNA transcripts of all MEF2 members undergo alternative splicing, with some of these being limited to specific cell types (Yu, 1996). The significance of these alternative splicing events is not yet understood.

The analysis of human tissues has shown that MEF2A is expressed only in skeletal and cardiac muscle and in the brain, while two isoforms of MEF2A that lack a beta domain region are expressed in all tissues examined (Pollock and Treisman, 1991). The duck MEF2A sequence cloned here was expressed in all three types of muscle, a finding similar to that for the two MEF2A isoforms in humans. We suggest that the duck MEF2A sequence cloned here may be only one of several alternatively spliced isoforms, and that the tissue expression of duck MEF2A may differ from that of other organisms. These possibilities warrant further study.

The modified sites in MEF2A are important for regulating gene expression and function. The phosphorylation status of MEF2A is a critical determinant of its tissue-specific functions and can be directly regulated by tissue-specific pathways acting on selected amino acid residues (Wang *et al.*, 2004). Pathways such as p38 MAPK signaling have an important role in regulating cardiac and smooth muscle function (Dentel *et al.*, 2005). As shown here, there are several important differences in the amino acid sequences of avian and mammalian MEF2A. Although the influence of amino acid alterations on MEF2A function, especially in the D1-D3 and V1 regions, was not examined here, these novel modified sites may be involved in the differential regulation of gene expression and function in avian and mammalian muscle.

In conclusion, duck MEF2A contains MADS, MEF2 and MAPK domains that share high similarity with related domains in other species. These conserved domains are important for maintaining protein functions. The expression of MEF2A in duck cardiac, smooth and skeletal muscle suggests an important role for this protein in muscle development.

Acknowledgments

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Internet Resources

CD-search tool available from NCBI, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

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