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Overexpression of MyoD Increased the Expression of RNA-Binding Proteins Rbm24 and Rbm38

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Abstract: MyoD can bind the E-box sequence (CANNTG) in the promoters of downstream muscle target genes such as myogenin thereby regulating their transcription. The expression of myogenic regulatory factors is promoted by transcription of their corresponding genes and post-transcriptional regulation during which RNA-binding proteins have an important role. However, a post-transcriptional modulator of MyoD has not yet been reported. In the present study, a duck eukaryotic expression plasmid pEGFP-N1-MyoD was successfully constructed and expressed in duck myoblasts. After transfection, the number of cells and cellular myogenin expression were significantly increased. Furthermore, the expression of Rbm24 and Rbm38 mRNAs was significantly increased. These results suggest that overexpression of MyoD increased the expression of Rbm24 and Rbm38 during duck myoblast differentiation. These findings lay the foundations for further research of the post-transcriptional regulatory mechanisms of MyoD.

Key words: MyoD, pEGFP-N1-MyoD, Rbm24, Rbm38, post-transcriptional modulator

INTRODUCTION

Myogenesis of skeletal muscle cells is a highly ordered process. Vertebrate skeletal muscles are derived from myogenic progenitor cells which are present in the somites. These progenitor cells are specified as myoblasts which firstly exit from the cell cycle and fuse with each other to form multinucleated myotubes, finally differentiate into skeletal muscle fibers (Buckingham, 2006). These transition steps are mainly controlled by Myogenic Regulatory Factors (MRF): MyoD, Myf5, myogenin and MRF4 (Buckingham, 2006). In mammals, MyoD and Myf5 are earlier markers of muscle development (Venters *et al*., 1999). When they are missing, myogenesis can not be initiated normally (Dezan *et al*., 1999). MyoD regulates target genes through promoter-specific transcriptional activation (Tapscott, 2005). For example, MyoD can regulate myogenin through interacting with the Pbx/Meis complex at the myogenin promoter region (Berkes *et al*., 2004). What's more, MyoD can also active p21, a Cyclin-Dependent Kinase (CDK) inhibitor which also play key roles in the process of myogenic differentiation (Halevy *et al*., 1995).

The expression of myogenic regulatory factors is not only due to the transcription of their corresponding genes but also due to the stabilization of their mRNAs (Bisbal *et al*., 2000) and the stability of mRNA is recognized as a critical post-transcriptional mechanism that controls the expression of a great number of genes. RNA-binding proteins play an important role in regulating gene expression in a post-transcriptional manner (Bolognani and Perrone-Bizzozero, 2008). The post-transcriptional mechanisms are generally believed to involve RNA-binding proteins that recognize specific RNA sequences which are known to be associated with AU-Rich Elements (AREs) in the 3'-Untranslated Region (3'-UTR) of labile mRNAs (Chen and Shyu, 1995; Xu *et al*., 1997). For example, Hu antigen R which contains the RNA Recognition Motif (RRM) is known to bind the 3'-UTR of MyoD, myogenin and p21 mRNA and it can contribute to myogenic differentiation by stabilizing the mRNA of these factors (Figureueroa *et al*., 2003). Therefore, the RNA-binding proteins play a greatly important role in preventing the degradation of mRNAs, consequently, maintaining the integrity of mRNAs.

In previous studies, the RNA-binding motif protein 24 (Rbm24) was found to be particularly expressed in cardiac and skeletal muscle tissues (Miyamoto *et al*., 2009; Terami *et al*., 2007). Xenopus tropicalis Rbm24 regulatory region contains E-box sequence which is potential binding site for MyoD and other MRF proteins

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(Li *et al*., 2010). Moreover, mouse Rbm24 has one RRM which is known to bind the 3'-UTR of myogenin and other MRF mRNAs. What's more, Rbm24 has been shown to be identified as a direct target gene of MyoD and regulate myogenin mRNA stability (Jin *et al*., 2010; Li *et al*., 2010). These data suggest that Rbm24 may not only be controlled by MyoD but also regulate myogenin during muscle cell differentiation. RNA-binding motif protein 38 (Rbm38) which shares a significant similarity with Rbm24 has been shown to bind to the 3'-UTR of the p21 transcript and regulate its stability (Shu *et al*., 2006). Thus, it would be kindly hypothesized that MyoD may regulate myogenin and p21 mRNA at least in part through Rbm24-dependent and Rbm38-dependent post-transcriptional regulatory pathways.

In this study, a duck eukaryotic expression plasmid pEGFP-N1-MyoD was constructed and the effects of MyoD-overexpressing on the expression of myogenin, Rbm24 and Rbm38 in duck were investigated. These findings may provide preliminary insights into the post-transcriptional regulatory mechanism of duck MyoD.

MATERIALS AND METHODS

Construct for the expression of MyoD

Gene amplification, cloning and sequencing: The full length cDNA of the duck *MyoD* gene consists of 894 bp (NCBI Reference Sequence: FJ374143.1). Expression vector pEGFP-N1-MyoD was constructed as follows. cDNAs encoding MyoD was amplified by RT-PCR with the following primers: the sense primer P1 5'-CCGGAAT TCATGGACTTACTGGGCGCCATGG-3' consisting of a EcoRI site and the antisense primer P2 5'-CGCGGATC CTTATAGCACTTGGTAGATAGGGTTGCTG-3' consisting of a BamHI site. The procedure for PCR was as follows: pre-denaturation reaction at 95°C for 5 min, 36 cycles consisting of denaturation at 95°C for 30 sec, annealing at 58°C for 40 sec, extension at 75°C for 40 sec and a final extension at 75°C for 10 min. The PCR products were separated by electrophoresis using 1.5% agarose gels and the target fragments were purified by gel extraction kit (Watson Biomedical Inc., Shanghai, China).

Subcloning: The purified target fragments were inserted into the plasmid pMD19-T and then transformed into competent *E. coli* DH5α cells. The recombinant plasmid was subjected to digestion with EcoRI and BamHI and then agarose gel electrophoresis was conducted to confirm whether the plasmids had proper gene inserts.

Both the recombinant plasmid and the pEGFP plasmid were double digested by EcoRI and BamHI, respectively. The target fragments were recovered and ligated using T4 DNA ligase (TaKaRa, Japan). The second recombinant plasmid was subsequently transformed into competent *E. coli* DH5α cells and extracted by DNA purification kit. The plasmid was also double digested by EcoRI and BamHI to evaluate its quality. Finally, the recombinant eukaryotic expression plasmid was named pEGFP-N1-MyoD (Fig. 1).

Cell culture: For each experiment, ten Pekin duck eggs incubated for 13 days were selected by chance. Muscle myoblast cells were isolated from the leg muscles of duck embryos according to the method of Lingli *et al*. (Sun *et al*., 2013). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich Japan, Tokyo, Japan) containing 15% Fetal Bovine Serum (FBS) and antibiotics (100 U mL^{-1} penicillin and 100 g mL^{-1} streptomycin). Cells were passaged at a split ratio of 1:2 when they covered most of the flask bottom. All procedures in the current study were approved by the Animal Welfare Committee of Sichuan province.

Gene over-expression: Growing myoblasts (70-80% confluent) were transfected with pEGFP-N1-MyoD or the pEGFP-N1 empty vector by using lipofectin 2000 (Invitrogen, USA) according to the manufacturer's instructions. At 12, 24 and 36 after transfection, cell numbers and the expression of green fluorescence protein

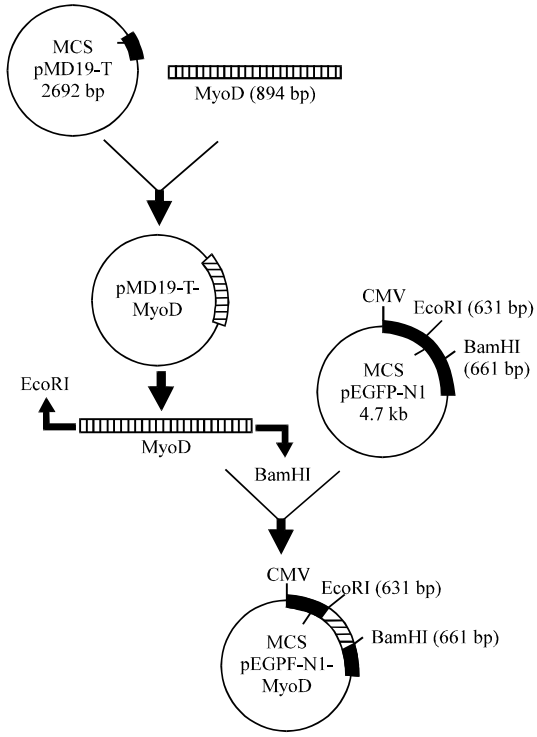

Fig. 1: Schematic representation for the construction of plasmid pEGFP-N1-MyoD

were measured by an inverted fluorescent microscopy to determine the transfection efficiency of pEGFP-N1-MyoD. Cell counting was processed by Image-Pro Plus Software. Finally, cells were harvested for RNA extraction. All experiments were performed three times in triplicate.

RNA extraction and real-time PCR: Total RNA was extracted from the cells of one 35 mm well (6 well plates) by Trizol (Invitrogen, USA) according to the manufacturer's instructions and then measured by spectrophotometer. RNA was reverse-transcribed to synthesis the cDNA by using the reverse transcript system (Takara, Japan). Quantitative Real-Time PCR (QRT-PCR) was carried out with SYBR Prime Script RT-PCR kit (TaKaRa, Japan) using the Bio-Rad CFX Manager (USA). One sample collected from leg muscles or cells was repeated 3 times. Real-time PCR was carried out under the conditions: pre-denaturation reaction at 95°C for 30 sec, 36 cycles consisting of 95°C for 10 sec and 60°C for 40 sec. The real-time PCR primers were designed (Table 1) for duck Rbm24 (NCBI Reference Sequence: KF542654), Rbm38 (NCBI Reference Sequence: KF542655), myogenin (NCBI Reference Sequence: GQ303573.1), MyoD and for GADPH (NCBI Reference Sequence: AY436595) and β-actin (NCBI Reference Sequence: EF667345). Duck GADPH and β-actin were used as internal controls. The data were calculated by the normalized relative quantification method followed by $2^{-\Delta\Delta C}$T (Livak and Schmittgen, 2001).

Statistic analysis: All data were presented as mean±SD. Differences between groups were analyzed by Analysis Of Variance (ANOVA) and values of $p<0.01$ were denoted statistical significance. All statistical analyses were performed using SAS (SAS Institute, Cary, NC, USA).

Table 1: Primers for QRT-PCR

Genes	Primer sequence
MyoD	
Forward	5'-AGGATTTCCACAGACAACTCCAC-3'
Reverse	5'-TTTGGGCTCCGCTATCATTC-3'
Myogenin	
Forward	5'-CGGATCACCTCCTGCCTGA-3'
Reverse	5'-CGTCCTCTACGGCGATGCT-3'
β-actin	
Forward	5'-GCTATGTCGCCCTGGATTTC-3'
Reverse	5'-CACAGGACTCCATACCCAAGAA-3'
GADPH	
Forward	5'-AAGGCTGAGAATGGGAAAC-3'
Reverse	5'-TTCAGGGACTTGTCATACTTC-3'
Rbm24	
Forward	5'-TTTGCCTTTGGTGTTCAGC-3'
Reverse	5'-CGATGTAGGGCGTAGTGGA-3'
Rbm38	
Forward	5'-CTATCATTCAGCCCAGCGTG-3'
Reverse	5'-CCCGTGTAGCCGTATCCC-3'

RESULTS AND DISCUSSION

Identification of recombinant plasmid pEGFP-N1-MyoD: The pEGFP-N1-MyoD plasmid was successfully constructed as confirmed by EcoRI and BamHI digestion and electrophoresis. The product of full-length *MyoD* gene digested from pEGFP-N1-MyoD plasmids was 894 bp which matched the expected size well (Fig. 2).

The transfection of pEGFP-N1-MyoD: The green fluorescence protein and mRNA expression were respectively analyzed by fluorescence microscopy and real-time PCR. After transfection for 24 h, a great number of myoblasts with GFP were visible (Fig. 3a). The real-time PCR results showed that the level of MyoD mRNA transfected with pEGFP-N1-MyoD plasmid was significantly increased at 24 and 36 h after transfection compared with the pEGFP-N1 group and control group ($p<0.01$) which peaked at 24 h (Fig. 3b). Above results indicate that the pEGFP-N1-MyoD plasmid was successfully expressed in duck myoblasts.

Effects of of MyoD-overexpressing on the expression of myogenin, Rbm24 and Rbm38: To evaluate whether overexpression of MyoD would affect myoblast proliferation and differentiation researchers observed the number of cells and the mRNA expression of myogenin at

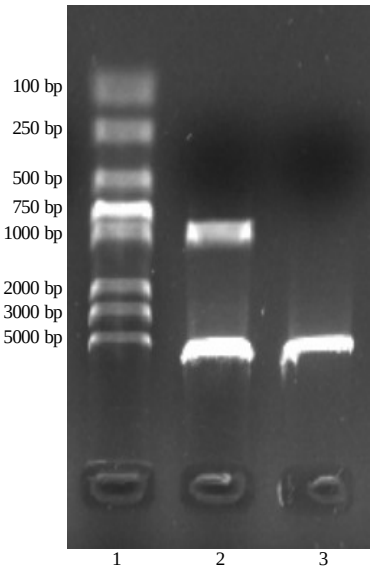

Fig. 2: Identification of the recombinant pEGFP-N1-MyoD plasmid. Lane 1: DNA marker; Lane 2: pEGFP-N1-MyoD plasmid double digested with EcoRI and BamHI. The larger band in lane 2 is pEGFP-N1 plasmid (4.7 kb) and the other band is *MyoD* gene (894 bp); Lane 3: the pEGFP-N1 empty

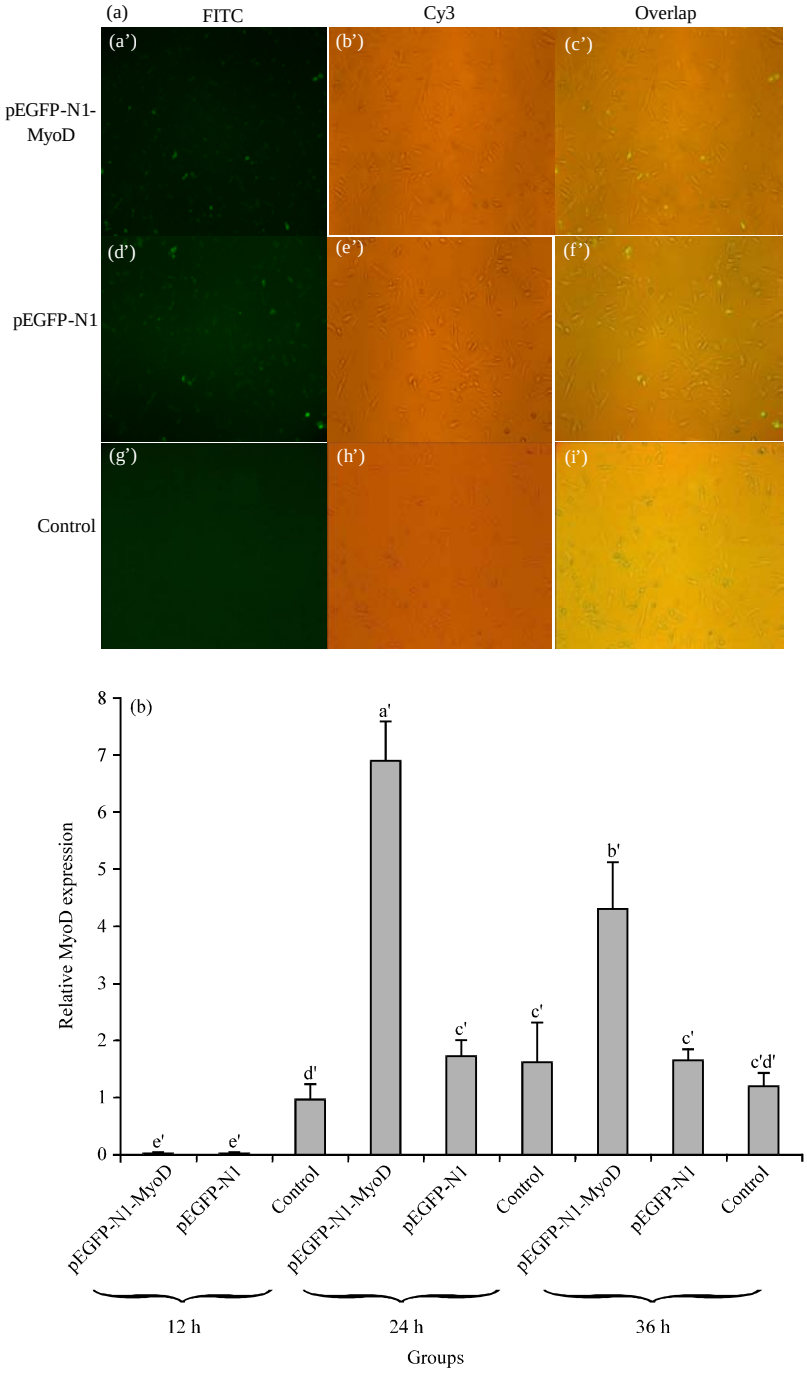

Fig. 3: Expression of recombinant plasmid pEGFP-N1-MyoD in duck myoblasts. a) The expression of Green Fluorescence Protein (GFP) in myoblasts at 24 h after transfection, detected by fluorescence microscopy (x100); a', b 'and c' show pEGFP-N1-MyoD-transfected myoblasts (pEGFP-N1-MyoD); d', e' and f' show pEGFP-N1-transfected myoblasts (pEGFP-N1); g', h' and i' show non-transfected myoblasts (Control); a', d' and g' indicate the GFP under Fluorescein Isothiocyanate (FITC); b', e' and h' show the morphology of the myoblasts under Cyanine Dyes 3 (Cy3); c', f' and i' reflect the overlap of two images in front. The cells contain GFP-identified positive cells. b) QRT-PCR showing MyoD mRNA expression in pEGFP-N1-MyoD, pEGFP-N1 and control cells at 12, 24 and 36 h after transfection

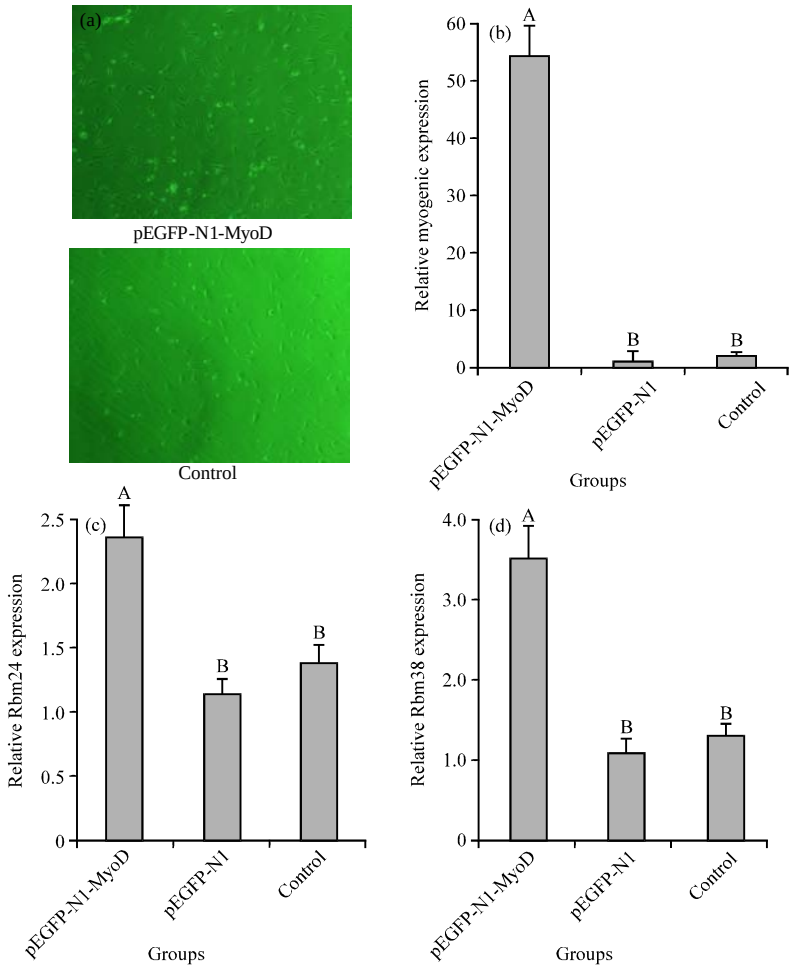

Fig. 4: Effects of MyoD-overexpressing on the expression of myogenin, Rbm24 and Rbm38. a) The image of myoblasts for the transfected cells compared to the control cells at 24 h after transfection; A and B indicate pEGFP-N1-MyoD-transfected myoblasts and non-transfected myoblasts, respectively. b-d) the mRNA levels of myogenin, Rbm24, Rbm38 in pEGFP-N1-MyoD, pEGFP-N1 and control cells at 24 h after transfection, detected by QRT-PCR. Different letters indicate a highly significant difference ($p<0.01$) in gene expression at different embryonic days whereas the same letters indicate no difference. Error bars indicate standard deviation

plasmid 24 h after transfection by microscopy and real-time PCR, respectively. The results showed that the number of cells in the pEGFP-N1-MyoD transfected group was larger than that in the control group (Fig. 4a) and expression of myogenin mRNA significantly increased after transfection (Fig. 4b).

In addition, overexpression of *MyoD* gene leaded to higher expression of Rbm24 and Rbm38 in which the mRNA expression level of Rbm24 and Rbm38 was increased >1.5 and 2 fold, respectively (Fig. 4c and d). These finding suggest that Rbm24 and Rbm38 may be the target genes of MyoD.

Previous studies have well established that MyoD played a central role in myogenesis which binded the simple core consensus sequence of CANNTG in promoters of downstream muscle target genes to regulate the transcription of these muscle-related genes such as myogenin (Puri and Sartorelli, 2000). In addition, MyoD would induce terminal cell cycle arrest during skeletal muscle differentiation by increasing the expression of p21 (Halevy *et al*., 1995). However, the post-transcriptional modulator of MyoD has not been reported.

RNA-binding proteins regulate gene expression through many events including splicing, export, stability, localization and translation (Keene, 2007). A previous study showed that Rbm24 (Seb4) was identified as a direct target gene of MyoD and was required for the function and expression of MyoD during myogenesis (Li *et al*.,

2010), furthermore, Rbm24 could also regulate myogenin expression (Jin *et al*., 2010). Rbm38 which is significantly similar to Rbm24, plays an important role in cell cycle arrest and myogenic differentiation via its binding to p21 (Miyamoto *et al*., 2009). Therefore, researchers can make a hypothesis, the post-transcriptional modulator of MyoD is carried out through the regulation of Rbm24 and Rbm38.

In present study, researchers construct a recombinant eukaryotic expression plasmid, pEGFP-N1-MyoD. pEGFP-N1 contains the Enhanced Green Fluorescent Protein (EGFP) and a single promoter which allows for translation of the gene of interest and EGFP from the same mRNA. Thus, the recombinant pEGFP-N1-MyoD plasmid can express both EGFP and MyoD protein. The transfection efficiency of pEGFP-N1-MyoD can be observed by fluorescence. After the recombinant plasmid was transfected into the duck myoblasts, the overexpression of MyoD mRNA was detected by RT-PCR, suggesting *MyoD* gene was successfully transfected into the duck myoblast. The data also demonstrated that the number of cells and the mRNA expression of myogenin were significantly increased by overexpression of MyoD which is consistent with previous results (Nabeshima *et al*., 1993). Interestingly, the mRNA levels of Rbm24 and Rbm38 were also significantly increased, suggesting that Rbm24 and Rbm38 may be regulated by MyoD. These data support the hypothesis that MyoD may regulate myogenin and p21 mRNA at least in part through Rbm24-dependent and Rbm38-dependent post-transcriptional regulatory pathways.

CONCLUSION

Researchers have successfully constructed the recombinant eukaryotic expression plasmid pEGFP-N1-MyoD. The *MyoD* gene was effectively expressed in duck myoblast and overexpression of duck MyoD not only increased the expression of myogenin but also increased the expression of Rbm24 and Rbm38. These findings may lay foundations for further research of the post-transcriptional regulatory mechanisms of MyoD.

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