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Promoter analysis of bovine cardiomyopathy-associated protein 1 gene

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ABSTRACT. The CMYA1 (cardiomyopathy-associated protein 1) is an actin-binding protein that plays a vital role in cardiac morphogenesis. CMYA1 is expressed specifically in the myocardial and skeletal muscle and is up-regulated in injured muscle. We therefore speculated that the bovine CMYA1 promoter might be muscle-specific. In this study, the promoter (+20/-1135) region of the bovine CMYA1 gene was cloned into a pEGFP-1 vector, and we found that the EGFP was observed only in C2C12 and myoblast cells. Thus, the CMYA1 promoter is muscle-specific. Thereafter, eight pGL3-basic vectors with various truncated CMYA1 promoter fragments were transfected into C2C12 cells, to identify the core promoter region using a Dual-Luciferase Reporter Assay System. The results showed that the promoter region from -457 to +20 bp was essential for CMYA1 to maintain the promoter activity, implying that this region may be the CMYA1 core promoter. We thus illustrate that the core promoter is muscle-specific. To evaluate the activity of the CMYA1 core promoter, the CMYA1 core and muscle creatine kinase (MCK) promoters were cloned into a pcDNA3.1 vector. The expression levels of their target genes were measured in C2C12 cells using real-time polymerase chain reaction. The CMYA1 promoter

drove the expression of the target gene six times higher than did the MCK promoter. The results thus suggest that the CMYA1 promoter could be an effective muscle-specific promoter, which may be useful in further studies of cardiomyopathy treatment and transgenic animal research.

Key words: CMYA1; Cardiomyopathy; C2C12 cells; Core promoter; Fragments

INTRODUCTION

The cardiomyopathy-associated protein 1 (CMYA1, also termed Xin), which was first obtained from chicken heart using differential mRNA expression analysis, regulates cardiac morphogenesis through a BMP-Nkx2.5-MEF2C pathway (Wang et al., 1996, 1999). CMYA1 is necessary for skeletal muscle formation and myocardium shape. It is located in the adhesion splice of the intercalated disc and the tendon-combining site of skeletal muscle (Jung-Ching Lin et al., 2005). CMYA1 has two exons, the second of which includes the whole coding region of the CMYA1 protein (Otten et al., 2010). Choi et al. (2007) used pull-down co-immunoprecipitation and yeast two-hybrid assays, to reveal that mouse CMYA1 directly interacts with β-catenin. The β-catenin binding site on mouse CMYA1 mapped to amino acids 535 to 636 (Choi et al., 2007). The CMYA1 gene contains a 16-amino acid repeat unit, a positioning signal, an SH3 genetic sequence combination, and a proline-rich domain (Wang et al., 1999). Nkx2.5, which is an important transcription factor of the CMYA1 gene, plays a crucial role in regulating CMYA1 gene expression. Analysis of Nkx2.5 knockout mice showed that CMYA1 expression was significantly lower than controls (Jung-Ching Lin et al., 2005). Both chicken and mouse CMYA1 can be regulated by both MEF2C and Nkx2.5 (Gustafson-Wagner et al., 2007). CMYA1 knockout mice are able to both survive and reproduce, which might be explained by compensatory action of CMYA3. However, the deletion of mouse CMYA1 leads to excessive heart growth and conduction blocks (Choi et al., 2007). It is thought that mouse CMYA1 and CMYA3 have different functions. A homozygous CMYA1 deletion mouse model was found to have disordered myocardium, abnormally sized heart, lack of ventricular septum, and diastolic dysfunction (Otten et al., 2010). The mouse CMYA1 plays an important role in the control of postnatal heart growth and, thus, in animal survival, by regulating N-cadherin (Wang et al., 2010). When CMYA1 expression was reduced, skeletal muscle regeneration was delayed (Nissar et al., 2012). The CMYA1 repeat-containing protein is beneficial for postnatal heart growth, as it promotes the maturation of the intercalated discs (Wang et al., 2013). In myopathies, CMYA1 is a marker for the severity of the skeletal muscle damage. The level of muscle damage and CMYA1 immunoreactivity are also strongly correlated, which suggests that CMYA1 may be an appropriate biomarker to evaluate disease progression and treatment effects in clinical trials (Nilsson et al., 2013). The CMYA1-repeat proteins are novel ligands of the SH3 domains of nebulin and nebulette. Furthermore, during muscle damage, the CMYA1-repeat proteins play a role in myofibril assembly (Eulitz et al., 2013).

Many studies have explored the role of the CMYA1 gene, but the promoter leading to its expression in the muscle has, to our knowledge, never been studied. As a critical cis-regulatory element for gene transcription, the promoter can drive both cell- and tissue-specific gene expression. At present, little is known about the bovine CMYA1 gene and its

promoter. Some studies have investigated the number of muscle-specific promoters, such as skeletal muscle α-action, muscle creatine kinase (MCK), myosin heavy chain, and so on. The relationship between the promoters and the muscle proliferation and transcriptional activity of these promoters has also been investigated (Skarli et al., 1998; Hagstrom et al., 2000; Zhu et al., 2005). In this study, we showed that the promoter and core promoter of CMYA1 are muscle-specific promoters. We also determined the core promoter region of the bovine CMYA1 gene, using a dual-luciferase reporter system. Our results illustrate that the core promoter region of the bovine CMYA1 gene is located at -457 to +20 bp. We also found that the CMYA1 promoter drives the expression of the target gene six times higher than the MCK gene promoter. The result is useful in further studies of cardiomyopathy treatment and transgenic animal research.

MATERIAL AND METHODS

All experimental protocols used in this study were approved by the Animal Care and Ethics Committee of Tianjin Agriculture University, Tianjin, China.

Sample collection

Nine cardiac muscle tissue samples were collected from three mature Simmental cross breeds and stored in liquid nitrogen until further analysis. Genomic DNA was isolated from the samples. The 1155 bp upstream of the CMYA1 gene 5'-flanking region, which covers the CMYA1 promoter sequence, was amplified from genomic DNA and restriction enzyme sites were added using the primer pair P1F and P1R (*Xho*I/*Hin*dIII; Table 1). Subsequently, the sequence was cloned into a pMD18-T vector (TaKaRa BIO, Tokyo, Japan) and confirmed by sequencing.

Vector construction

In order to identify the core promoter of CMYA1, we constructed a dual-luciferase reporter including truncated CMYA1 promoter fragments with different sizes. In addition, eight pairs of primers were designed to amplify eight fragments with the pMD18-T plasmid (TaKaRa BIO) as template. The 5'-ends of the forward primers were added using the *Xho*I restriction site and the 5'-ends of reverse primers were added using the *Hin*dIII restriction site (the primer information is shown in Table 1). The fragments were cloned into the pMD18-T vector. The fragments were then excised from the pMD18-T vector using *Xho*I and *Hin*dIII restriction endonucleases and subcloned into the promoter vector pGL3-basic (Promega, Madison, WI, USA). The recombinant constructs were named pGL3-basic-P1 (+20/-1135), pGL3-basic-P2 (+20/-982), pGL3-basic-P3 (+20/-932), pGL3-basic-P4 (+20/-817), pGL3-basic-P5 (+20/-670), pGL3-basic-P6 (+20/-553), pGL3-basic-P7 (+20/-457), and pGL3-basic-P8 (+20/-323). In order to explore the muscle-specificity of the CMYA1 promoter, we used the same method as described above to subclone the fragments into a pEGFP-1 vector (pEGFP-1-CMYA1-P1-P8).

To investigate the activity of the CMYA1 core promoter, we used standard recombinant DNA methods to produce two eukaryotic expression vectors called pcDNA-MCK-DGAT1 and pcDNA-CMYA1-DGAT1. The eukaryotic expression vector pcDNA3.1 (Invitrogen, USA) was used as a framework. The CMYA1 promoter and the MCK promoter were both

cloned into the pcDNA3.1 vector. The pcDNA-MCK-DGAT1 expression vector contained mouse MCK promoter and bovine DGAT1 (diacylglycerol acyltransferase 1) cDNA. The construction process was as follows: first, *Mlu*I/*Hin*dIII restriction enzymes were used to digest the pcDNA3.1 vector and MCK (synthesized by the GENEWIZ Inc. Company). Junction fragments of the enzyme-digested products were then used to obtain the intermediate vector pcDNA-MCK. *Eco*RI/*Eco*RV restriction enzymes were then used to digest pcDNA-MCK and DGAT1 CDS (GenBank accession No. NM_174693). Finally, the junction fragments of the enzyme-digested products were used to obtain the final expression vector pcDNA-MCK-DGAT1. The resulting product was identified using PCR and sequencing. In the same manner, the pcDNA-CMYA1-DGAT1 expression vector was constructed, but the MCK promoter was replaced by the CMYA1 core promoter (Table 1).

Cell culture and transfection

Mouse myoblast cells (C2C12), bovine myoblast cells, and bovine fibroblasts cells (stored in our laboratory) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 15% fetal bovine serum (FBS, Gibco). The cells were incubated at 37°C under 5% CO_2. Before transfection, the bovine myoblast cells, bovine fibroblasts cells, and C2C12 cells were seeded at 5 x 10^6 cells/dish on a 3.5-cm culture dish and cultured overnight. Subsequently, the transient transfection was performed with the promoter constructs using Lipofectamine™ 2000 (Invitrogen). Each plate was transfected using 3:1 Lipofectamine™ 2000 to total plasmid DNA. Cells were transfected in triplicate, for each construct. When the transfections were performed using the luciferase reporter construct, the ratio of internal control plasmid for *Renilla* luciferase expression, PRL-TK vector (Promega) to promoter luciferase reporter plasmids or positive control plasmid pGL3-control vector (Promega) was 1:30.

Double-fluorescein system testing

Following 24-26-h transfection, the cells were lysed for 35 min. *Renilla* luciferase and firefly luciferase activities were analyzed using the Dual-Luciferase® Reporter Assay System (Promega). The firefly luciferase activities were normalized using the *Renilla* luciferase activity in each dish and luciferase vector pGL3-basic values were used as negative controls.

Relative quantity PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, No. 15596-026) and left for 10 min; RNA was separated in chloroform by centrifugation at 12,000 *g* for 15 min. It was then precipitated in isopropanol and eluted by ethanol. The RNA was then dissolved in diethylpyrocarbonate-treated water. Total RNA was reverse-transcribed into cDNA. The relative quantity PCR was tested using the CFX96™ and CFX384™ real-time detection systems and the comparative Ct value method ($2^{-\Delta\Delta Ct}$). The primers used are listed in Table 1.

Statistical analysis

The statistical evaluation of the data was conducted using SPSS v. 19.0 (APSS,

Chicago, USA) One-way ANOVA and two-tailed *t*-tests were used for the statistical analyses. Data are reported as means ± SD. A statistical significance level of $P \leq 0.05$ was used in all cases.

RESULTS

Tissue-specificity

In order to explore the muscle-specific of the CMYA1 promoter, the pEGFP-1-CMYA1-P1 vectors were transfected into C2C12 cells, bovine myoblast cells and bovine fibroblasts cells, respectively. EGFP emits fluorescent light that can be detected with a DMLB microscope (Leica Microsystems GmbH, Wetzlar, Germany) with appropriate filter sets. EGFP was expressed in C2C12 cells and in bovine myoblast cells, whereas very little expression was observed in bovine fibroblast cells. Thus, the full-length promoter sequence is muscle-specific and showed high expression activity (Figure 1).

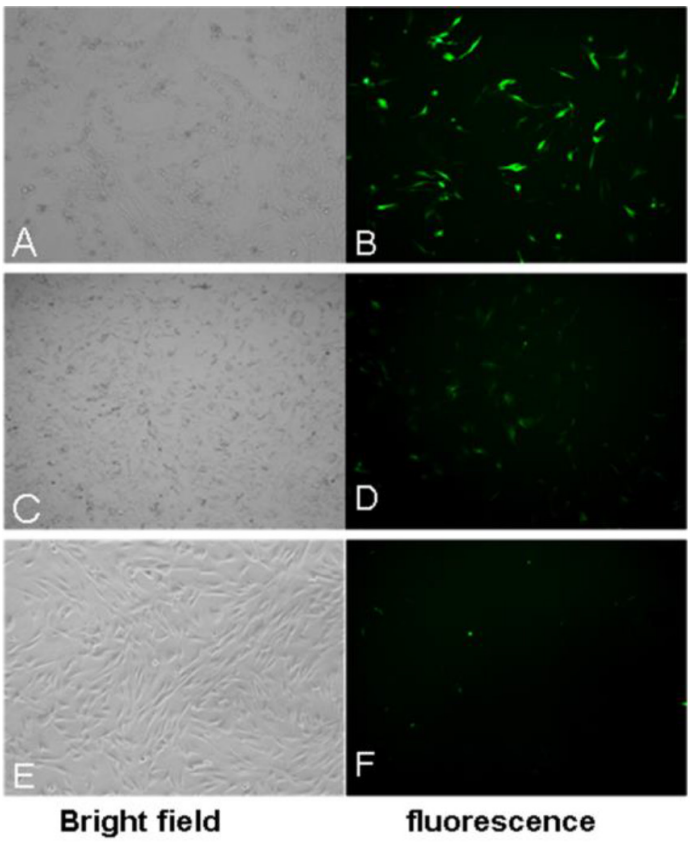

Figure 1. pEGFP-1-CMYA1-P1 was transfected into C2C12 cells, bovine myoblast cells, and bovine fibroblasts cells. **A.** and **B.** EGFP expression in C2C12 cells. **C.** and **D.** EGFP expression in bovine myoblast cells. **E.** and **F.** EGFP expression in bovine fibroblasts cells. All figures show expression at 24 h after transfection and a 100X magnification.

Identification of the CMYA1 core promoter

To investigate the promoter activity of the bovine CMYA1 gene, the luciferase reporter constructs containing truncated CMYA1 promoter fragments with different sizes (pGL3-basic-P1-P8) were transiently transfected into C2C12 cells. The luciferase activity derived from the differently sized CMYA1 promoters was determined using the Dual-Luciferase® Reporter Assay System. The luciferase activities of all the luciferase reporter constructs, except pGL3-basic-P8 (+20/-323), were significantly higher compared to the pGL3-basic negative control (Figure 2). A significant increase in luciferase activity was observed in pGL3-basic-P7 (+20/-457) compared with pGL3-basic-P8 (+20/-323) and the luciferase activities maintained high levels with increasing promoter fragment lengths at all time points. This suggests that the essential core region of the CMYA1 promoter is located at -457 to +20 bp. Furthermore, several promoter regions, including pGL3-basic-P6 (+20/-553) and pGL3-basic-P7 (+20/-457), showed higher luciferase activities than the pGL3-control positive control (Figure 2).

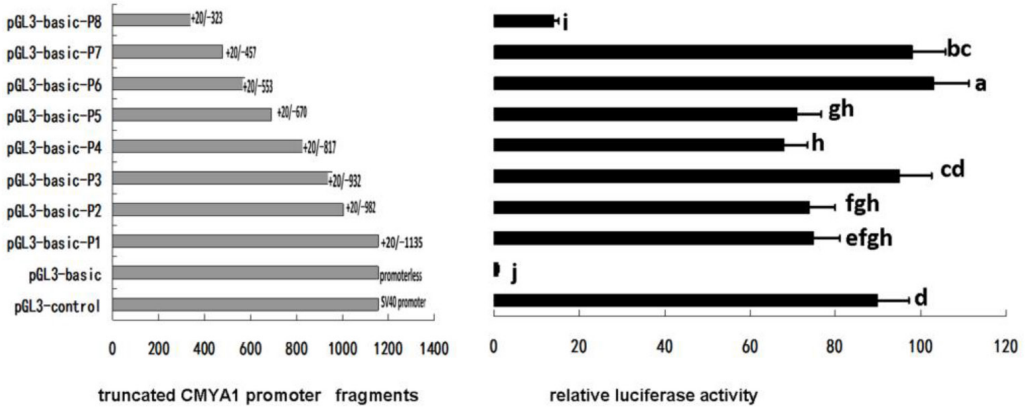

Figure 2. Identification of the CMYA1 core promoter. The left panel represents a series of truncated 5'-flanking sequences of the CMYA1 gene, whereas the right panel represents the relative luciferase activities of the promoter fragments in the C2C12 cells. The pRL-TK vector encoded *Renilla* luciferase and was co-transfected as an internal reference. One-way ANOVA test was used to analyze differences in luciferase activity. The values are reported as means ± SD of three independent replicates. Different lower case letters indicate significant differences at $P \leq 0.05$.

Tissue-specificity of the CMYA1 core promoter

In order to verify whether the CMYA1 core promoter was muscle-specific, the core promoter fragment pEGFP-1-CMYA1-P7 was transfected into C2C12 cells, bovine myoblast cells, and bovine fibroblasts cells. EGFP was found to be expressed in C2C12 cells and in bovine myoblast cells, whereas little expression was observed in bovine fibroblasts cells. These results suggest that, like the full-length promoter sequence, the bovine CMYA1 core promoter (sequence from -457 to +20 bp) was also muscle-specific (Figure 3).

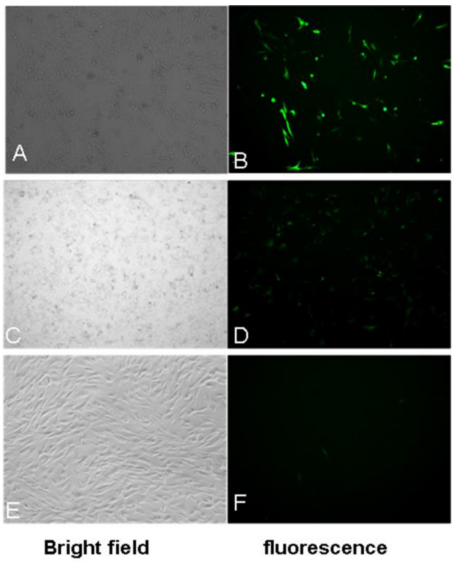

Figure 3. pEGFP-1-CMYA1-P7 was transfected into C2C12 cells, bovine myoblast cells, and bovine fibroblasts cells. **A.** and **B.** EGFP expression in C2C12 cells. **C.** and **D.** EGFP expression in bovine myoblast cells. **E.** and **F.** EGFP expression in bovine fibroblasts cells. The figures show expression at 24 h after transfection and a 100X magnification.

Activity of the CMYA1 and MCK promoters

The pcDNA-CMYA1-DGAT1 and the pcDNA-MCK-DGAT1 vectors were transiently transfected into C2C12 cells. Total RNA was isolated from the C2C12 cells using traditional RNA isolation methods, after a 24-h transfection. The cDNA was reverse-transcribed from total RNA and the GAPDH gene was selected as the reference control gene. The primers for the DGAT1 and GAPDH genes are presented in Table 1. Compared to the blank control groups, the relative expression levels of DGAT1 in the pcDNA-CMYA1-DGAT1 and pcDNA-MCK-DGAT1 experimental groups were 30 and 5 times higher, respectively. In this experiment, we showed that the CMYA1 promoter drives the expression of the target gene six times higher compared to the MCK gene promoter (Figure 4).

Table 1. Primer pairs used in the experiment.

Primer name	Primer sequences (5'-3')	Annealing temperature (°C)	Time (s)	Size (bp)
Primers used in the promoter activity analysis				
P1F	ccgCTCGAGGGCCAATGCTGCCTGCCTGACGCTG	58	80	1155
P2F	ccgCTCGAGTGCCTCAGTCATTTGTGC	58	70	1002
P3F	ccgCTCGAGTGCCCTTCCCAGCCTAAA	58	60	952
P4F	ccgCTCGAGCTGTGACGGGAAGCTGAT	58	60	837
P5F	*ccg*CTCGAGGCTGTGAACACCGCAGAC	58	50	690
P6F	ccgCTCGAGTACCTGTGCGGTGGGATG	58	50	573
P7F	ccgCTCGAGGGGCTGGGAAATGAGGAG	58	50	477
P8F	ccgCTCGAGGGCCAAGTTGACCAGGTGT	58	40	343
PDR	ccc*AAGCTT*GCTGCCCTAGCTCGGGGTCT	-	-	-
Primers used in qRT-PCR				
GAPDH-F	CCAGGTGGTGTCTCTGTTCC	60	40	186
GAPDH-R	GCTGGGAAGCAGAGAATGGT			
DGAT1-F	GAAGGTCGGTGTGAACGGAT	60	40	186
DGAT1-R	TTCCCATTCTCGGCCTTGAC			

Restriction sites are underlined.

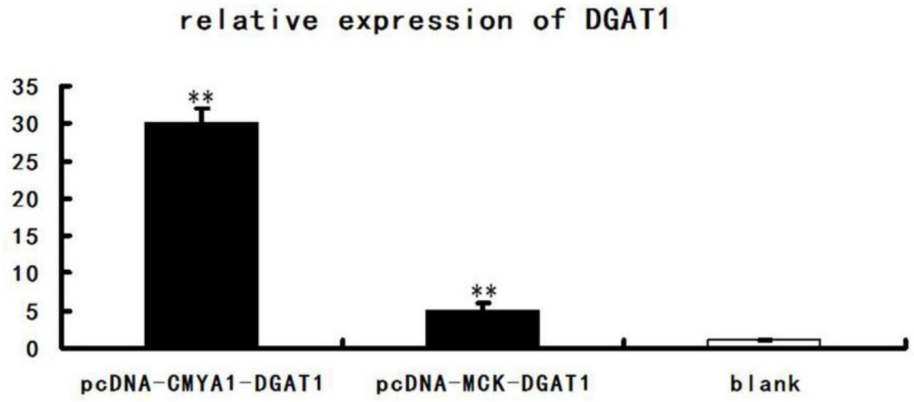

Figure 4. pcDNA-CMYA1-DGAT1 and pcDNA-MCK-DGAT1 vectors transiently transfected into C2C12 cells. The control group (blank), which was transfected in pcDNA3.1 vector, was given a relative value of 1. Two-tailed *t*-tests were used to analyze the difference in relative expression of DGAT1. Both experimental groups were significantly different from the control group. The values are reported as means ± SD of three independent replicates. **P < 0.01.

DISCUSSION

The CMYA1 is one of the components of the adherens junction complex in both sarcomere assembly and cardiac morphogenesis (Sinn et al., 2002; Jung-Ching Lin et al., 2005). Previously, the bovine CMYA1 gene has been shown to be expressed specifically in striated muscle tissue (Nie et al., 2013). The bovine CMYA1 mRNA sequence (6270 bp) has been deposited in the GenBank nucleotide database (accession No. HQ111436.1) (Nie et al., 2013). Based on the putative transcription factor binding site (Wu et al., 2013), we chose the 1155 bp (+20/-1135) upstream of the CMYA1 gene 5'-flanking region to analyze the core promoter. Some muscle-specific gene promoters can regulate gene expression in myocytes but not in fibroblasts (Salva et al., 2007). In the present study, pEGFP-1-CMYA1-P1 and pEGFP-1-CMYA1-P7 fragments were transfected into C2C12 cells, bovine myoblast cells, and bovine fibroblasts cells. The difference in promoter activity among the different cell lines implies that the CMYA1 promoter is muscle-specific (Figures 1 and 3). In this study, we showed that both the full-length bovine CMYA1 promoter sequence and the CMYA1 core promoter are active specifically in muscle tissue.

For gene transcription, the promoter is an important cis-regulatory element. In order to reveal the transcriptional regulatory mechanisms, cloning and analysis of the promoter are therefore important steps (Wu et al., 2013). We hope that this study may serve as a basis for further investigation of the detailed function of the CMYA1 core promoter.

In the study of transgenic animals, exogenous genes usually require specific expression in some organizations and the tissue-specific promoter plays an important role in this process. One of the bases of the study transgenic animals is to study effective tissue-specific promoters. The MCK gene is expressed at high levels in both skeletal and cardiac muscles (Johnson et al., 1989). Transcriptional regulation of the MCK gene has been shown in transgenic mice as well as in cultured myoblast cells (Johnson et al., 1989). The MCK gene promoter is one of the effective tissue-specific genes that is often used to study transgenic animals (Johnson et al.,

1989). The DGAT1 gene is a functional candidate gene for intramuscular fat content (Liu et al., 2007). The DGAT1 gene has also become a target for gene manipulation by transgenic or other routes, to modify various animal traits. In order to study whether the bovine DGAT1 gene could be expressed in muscle under the control of MCK promoter, a pcDNA-MCK-DGAT1 vector was constructed and its function was investigated in a transgenic mouse model (Yang et al., 2013). The transgenic model was established for the study of transgenic bovine with high intramuscular fat content. In the present study, a pcDNA-CMYA1-DGAT1 vector was constructed and we found that the CMYA1 promoter drove the expression of the target gene six times higher compared to the MCK promoter. The function of the CMYA1 promoter will be studied in a CMYA1-DGAT1 transgenic mouse model in the near future in our laboratory. We predict that the transgenic animal model will show a higher rate of muscular fat than the MCK-DGAT1 transgenic mice. The hope is that our study will serve as a basis for further studies of cardiomyopathy treatment and transgenic animal research.

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