Master's Thesis of Science in Agriculture

Study on Muscle Differentiation in Quail Myoblast Cells Genome-Edited by CRISPR-Cas9 System

CRISPR-Cas9 시스템으로 유전자를 교정한
메추리의 근원세포에서의 근육 분화 연구

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Abstract

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In the livestock industry, the regulatory mechanisms of muscle proliferation and differentiation are very important to understand skeletal muscle growth. This study is about to investigate the regulatory pathway of MyoD and its role in muscle differentiation in QM7 (quail muscle clone 7) myoblast cells. The MyoD gene was mutated by the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 technology and single cell-derived MyoD mutant sublines were identified to investigate the regulatory mechanism responsible for muscle differentiation. We induced the differentiation by changing medium without serum during cell culture. The mutation efficiency in the mixed population was 73.3%. We selected a single cell (MyoD KO QM7#4) from the mixed population and cultured to expansion. We confirmed the difference between on the 3, 6 day of differentiation. The expression of paired box 7 (Pax7), which is the undifferentiated marker, was not significantly different from regular QM7 (rQM7) cells to MyoD KO QM7#4 cells. However, there was a significant difference in morphology between the rQM7 cells and MyoD KO QM7#4 cells during

differentiation. The differentiation to myotube formation and the nuclear fusion rate was glaringly suppressed in MyoD KO QM7#4 cells comparing with rQM7 cells. And the myogenic differentiation-related genes also were not detected in MyoD KO QM7#4 cells during differentiation. In conclusion, we can assume that MyoD is a critical factor for muscle differentiation in the quail myoblast and the CRISPR/Cas9-mediated genomic editing can be adapted to various aspects, not only for this study, but also for functional genomic study in the poultry science.

Keywords **:** myoblast, CRISPR-Cas9, knockout, muscle differentiation, MyoD

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1. Introduction

1.1. Study about muscle growth

1.1.1. Importance of growth study in the industry

Research on muscle growth in human and agriculture research is vital, and has long been studied. In particular, research on animal growth in livestock fields is essential to the research sector because it is very important for selection and breeding (Hillier and Pachter 2004, Rubin, Zody et al. 2010). It is same in the poultry industry. Since the growth of skeletal muscle depends on the differentiation and proliferation of muscle cells, we need to understanding the mechanism of muscle differentiation and proliferation to know about skeletal muscle growth (Al-Musawi, Lock et al. 2011, Shin, Song et al. 2015).

1.1.2. Myogenic regulation-related factors

There are many genes associated with muscle growth in different species. The key transcription factor associated with the differentiation are called myogenic regulatory factors (MRFs). MRFs are basic helix-loop-helix (bHLH) transcription factors that regulate myogenesis. MyoD, Myf5, myogenin, and MRF4 are included in MRFs(Perry and Rudnick 2000). They are known expressed in the nuclear of satellite cells activated in the damaged muscle fibers and the nucleus in the muscle fiber during the muscle fibers are recycling (Rudnicki, Schnegelsberg et al. 1993). In particularly in the development of muscle,

myogenesis, including myoblast proliferation and embryonic muscle development, is promoted by 4 MRFs and suppressed by myostatin. MyoD and myogenin are known as an important factor for the determination of the myoblast myogenic progenitor specification (Stern, Ashwell et al. 2015).

Among the several myogenic regulation-related gene, MyoD and Pax7 have been well-known as play a major role in the differentiation of muscle. MyoD maintains differentiation and Pax7 maintains suppression of differentiation**.**

1.2. Backgrounds for this experiment

1.2.1. Quail muscle clone 7 (QM7) cell

The Quail muscle clone 7 (QM7) cell line was derived from the QT6 fibrosarcoma originally isolated by Moscovici, et al (Antin, Karp et al. 1991, Antin and Ordahl 1991). The cells replicate as myoblasts in medium containing serum. When switched to medium without serum, the cells cease dividing and fuse to form large multinucleated myotubes. In the myotube state, the cells express muscle specific proteins such as desmin, cardiac troponin T, cardiac troponin C, skeletal troponin T, skeletal troponin I, alpha tropomyosin. QM7 cells were transfected with high efficiency, and are useful for studying many aspects of muscle differentiation and gene expression.

1.2.2. CRISPR/Cas9 system for genomic editing

To determine the regulatory mechanisms of specific genes, we have to edit gene structure with transfer and knockout (Park and Han 2012, Park and Han 2012, Park, Lee et al. 2014). The piggybac transposon element has been used for efficient delivery of foreign transgenes into the hast genome of avian species, particularly in chickens (Salter, SMITH et al. 1986, Love, Gribbin et al. 1994, Macdonald, Taylor et al. 2012, Park and Han 2012). The specific genes can be removed from the sequence by knockout techniques (Schusser, Collarini et al. 2013, Park, Lee et al. 2014, Dimitrov, Pedersen et al. 2016). Clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas9-mediated genome editing technique has been developed and widely used to functional genomic study (Jao, Wente et al. 2013, Mali, Yang et al. 2013). It is a 3rd-generation programmable nuclease, following Zinc Finger Nuclease (ZFN, 1st-generation) and Transcriptor Activator-Like Effector Nucleases (TALENs, 2nd-generation) (Gaj, Gersbach et al. 2013).

It was the 1970s when genetic engineering first began. Starting with the discovery of a ' restriction enzyme ' that was detected by DNA, the gene engineering technique was born to cut, paste and insert DNA. However, the limitations of gene engineering using restriction enzymes were obvious. The length of the recognized sequence was too short. So there is a new way to replace restriction enzymes, which is gene programmable nuclease.

ZFN is combined with zinc finger and 3 to 4 nucleases. In the mid-1980s, while studying for African clawed frog, the scientists found hand-shaped DNA. They put the name ‘Zinc

finger' because there was a zinc in this gene. In 1996, the first programmble nuclease was disclosed, that is the combination of restriction enzyme called Fokl and six zinc fingers. Engineered zinc finger arrays are often combine with FokI, which is a DNA cleavage domain, to generate zinc finger nucleases. Zinc finger-FokI combinations have become useful reagents for genome editing of many higher organisms (Klug and Rhodes 1987) like zebrafish (Reynolds and Miller 1988), tobacco, corn (Shukla, Doyon et al. 2009), several mammalian cells (Carroll 2008), and rats (Geurts, Cost et al. 2009). Xenopus laevis TFIIIA was the first reported zinc requirement for a gene regulatory protein (Hanas, Hazuda et al. 1983, Berg 1990). 1 domain can reading 3 nucleotides, and cut sequences by combine with DNA binding domains. But ZFN system has some weaknesses such as high cytotoxicity and mutation rate.

So, the next genome editing system was appeared. The 2nd-system is TALENs(Transcriptor Activator-Like Effector Nucleases). TAL effectors were originated from Xanthomonas which cause plant disease (Boch and Bonas 2010). Transcription activator-like effectors (TALEs) can be engineered to bind to any DNA sequence. When the TALEs combined with a nuclease, DNA can be cut specifically (Boch 2011). If amino acids of TALENs change, sequences of the combined target also changed. In other words, change in protein could be easier. 1 TALE protein can recognize 1 nucleotide. TALEN can reading more bases and also cost less than ZFN, so it was able to go ahead. However, these systems are basically difficult to be designed and have low accuracy since it is hard to bind many proteins together.

And finally, more accurate and easy way to genome editing, CRISPR/Cas9 system was revealed. CRISPR/Cas9 system was originated from an immune system of bacteria which fight against virus using the sequence called ‘CRISPR’. CRISPR are short, repetitive base sequences of prokaryotic DNA. These sequence have a critical role in a bacterial defence immune system (Barrangou 2015). The sequence of nucleotides is same in both directions of a palindromic repeat. Each repetition contains short segments of spacer DNA from former exposures to exotic DNA like other virus or plasmid (Marraffini and Sontheimer 2010). The biggest difference between CRISPR and other prorammable nuclease, is protein replaced to RNA to find specific sequence. Protein was hard to operate because of a macromolecule, but we can recombinate a specific gene easily using RNA with high efficiency. CRISPR/Cas genome editing techniques can applied many sphere of genomic study in the science. Genome editing with CRISPR system will develop further for diverse field (Ledford 2016).

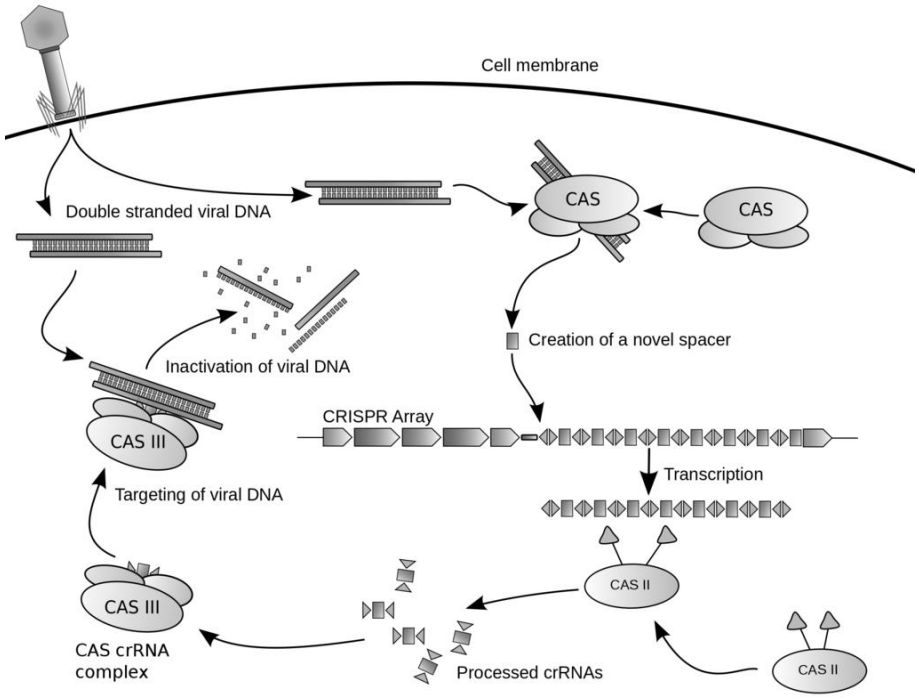

Figure 1. Mechanism of the CRISPR defence system (Horvath and Barrangou 2010).

1.2.3. String analysis

There was no genomic information on quail, we had to identify any functional networks in the differentiation process (Simpson, Joggerst et al. 2012). We used the string analysis by RNA sequencing in QM7 MyoD KO cells. Functional connection between proteins are often be analogized from genomic relations between genes that coding the proteins. A group of genes required for the same function tend to be presented a similar species. String analysis is a global way to be used to investigate and analyze these associations from the connections. Performing string analysis based on the RNA sequencing data, it can predict the expectation for any functional interactions (Mering, Huynen et al. 2003). Focusing on the lists of striking genes under differentiation pathway, we used the 'Trinotate' applying some options to reveal a number of networks.

1.2.4. The hypothesis of this study with MyoD

MyoD was the first identified myogenic regulatory-related gene (Davis, Weintraub et al. 1987). MyoD is expressed at extremely low level and undetected in quiescent satellite cells, but it is activated when the muscle exercise or muscle tissue damage (Berkes and Tapscott 2005, Buckingham 2006). MyoD is essential factor to transcript the DNA that coding muscle specific protein such as actin and myosin. After actin and myosin synthesized by MyoD protein, myoblast differentiate to myotube. Although, MyoD is one of the myogenic regulatory factors, it will not differentiate into myotube by only MyoD. MyoD is one of the early myogenic regulatory factors for determination and differentiation of skeletal muscle (Berkes and Tapscott 2005, Buckingham 2006). So we can assume that the decrease of MyoD during the early differentiation state, after proliferation, cause some influence with further differentiation. We assumed that knockout of MyoD would depress differentiation of myoblast into myotube, and conducted the experiment to confirm this hypothesis.

In this study, we knocked out the MyoD gene in quail myoblast cells by the CRISPR/Cas9 genomic editing system and ascertained the regulatory pathway of MyoD under the myotube differentiation state.

2. Materials and Methods

2.1. Quail Myoblast (QM7) cell culture and induction of myotube differentiation

QM7 cells(American Type Culture Collection, Manassas, VA, USA) were maintained at 37℃ in an atmosphere of 5% CO2 and 60% to 70% relative humidity with Medium 199 containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 2% chicken serum (Sigma-Aldrich, St. Louis, MO, USA), and 1× antibiotic-antimycotic (Invitrogen, USA) by subculturing the cells at 70% confluency. To induce myotube differentiation at 90% confluency, the differentiation medium containing 0.5% FBS and 1× antibiotic-antimycotic was changed and half of the medium was replaced with fresh differentiation medium daily.

2.2. CRISPR/Cas9-mediated MyoD knockout and fluorescence-activated cell sorting

For knockout of the MyoD gene, 7.5 μL Lipofectamine 3000 Reagent was diluted in 250 μL OPTI-MEM (Invitrogen, USA), and 2.5 μg each of the Cas9- green fluorescent protein (GFP) co-expression plasmid (Sigma-Aldrich, USA) and MyoD guide RNA (gRNA) was mixed with Lipofectamine P3000 Reagent in 250 μL OPTI-MEM at room temperature. After incubation for 5 min, the two mixtures were combined and incubated for an additional 20 min. The complex mixture was gently pipetted and dropped into a six-well plate containing QM7 cells at 70% to 80% confluency. After incubation at 37℃ in 5% CO2 for 4 h, cells were gently washed with phosphate-buffered saline (PBS) three times, and fresh culture medium

was added. One day after lipofection, GFP-expressing cells were sorted using a FACSAria III cell sorter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Following harvest using 0.05% trypsin-ethylenediaminetetraacetic acid (Invitrogen, USA), cells were resuspended in PBS containing 0.1% bovine serum albumin (BSA) and strained through a 40 μm cell strainer for fluorescence-activated cell sorting (FACS) (Becton, Dickinson and Company, USA). After sorting, the cells were regrown in culture media for subsequent experiments. To isolate single cell-derived sublines, each well-isolated single colony was isolated and subcultured using smooth silicone grease and a cloning cylinder.

2.3. Genotyping by T-vector cloning and sequencing

Genomic polymerase chain reaction (PCR) was performed using an initial incubation at 94℃ for 5 min, followed by cycles of denaturation, annealing, and extension for each target gene or locus using the corresponding primer sets (Table 1). The reaction was terminated with a final incubation at 72℃ for 7 min. To confirm the target locus mutation, PCR amplicons were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Table 1. List of primer sets for polymerase chain reaction analysis

Gene	Forward	Reverse	Annealing Temp.(℃)
GAPDH	TGTCAAGGCTGAGAACGGGA	AGGCCAGGGAGCATTCTTCT	60
MyoD	ATTGGCAATGAGAGGTTCAGG	TAGAGCCTCCAATCCAGACAGA	60
Myogenin	AGCCTCAACCAGCAGGAGC	TGCGCCAGCTCAGTTTTGGA	60
Desmin	CTGAAGGATGAGATGGCC	GGTCGCCTCGCTCACCAC	60

2.4. De novo assembly of RNA-sequencing data of quail transcripts

Gene expression level was estimated through the Trinity pipeline (Version 2.2.0), with RNA-sequencing by expectation maximization (RSEM) as the transcript quantification method. We employed the Trinity pipeline for non-model organisms, mapping the reads into an assembled consensus without genomic reference to measure transcriptome levels. Trinity allows for the identification of transcript isoforms in non-model species. Prior to assembly, we employed Trimmomatic (Version 0.35) for the removal of adapter sequences. Subsequently, we performed assembly and mapping as follows. To make the reference consensus and assemble the transcriptome, we combined left and right reads in paired-end reads using 18 samples. Then, using combined left and right reads, we created the assembled transcriptome with 'trinityrnaseq-2.2.0/Trinity' applying the default options. Also, we performed read mapping onto the assembled transcriptome (bowtie2) and abundance estimation (RSEM) using align_and_estimate_abundance.pl applying the '--est_method RSEM--aln_method bowtie--trinity_mode' options. Gene annotation was performed using TransDecoder, implemented within Trinity, and we applied several developer-recommended gene annotation tools such as blastx and blastp. Finally, using Trinotate, we extracted information on annotated genes applying the default options. To define known transcriptomic regions, we used Trinotate (http://trinotate.github.io) applying the default options.

2.5. Statistical analysis

Using the trimmed mean of M-values normalized expression as the response, the analysis of deviance model was employed to test for significance between the knockout (KO) and control groups, as follows:

$$Expression_i = \mu + Group_i \quad \text{(Eq. 1)}$$

where i = {control, KO} with control samples serving as the baseline. The negative-binomial assumption was considered a response variable to solve the over-dispersion problem in count data. Under the null hypothesis (H0: Group = 0), a likelihood ratio test was performed and the p-values were adjusted based on false discovery rate. Here, a 1% significance level was considered significant.

2.6. Reverse transcription PCR and quantitative reverse transcription-PCR

Total RNA from undifferentiated and differentiated QM7 and MyoD KO QM7#4 cells was isolated using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA quality was checked by agarose gel electrophoresis and quantity was determined with a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized from RNA using the Superscript III First-Strand Synthesis System (Invitrogen, USA). Each 20 μL reverse transcription-PCR (RT-PCR) reaction contained 2 μL cDNA, 2 μL PCR buffer, 1.6 μL dNTP mixture (2.5 mM), 1 unit Taq DNA polymerase, and 10 pmol forward and reverse primer (Table 1). PCR was performed with an initial incubation at 94℃ for 5 min, followed

by 35 cycles at 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 30 s. The reaction was terminated with a final incubation at 72℃ for 10 min and the products were analyzed by agarose gel electrophoresis. For quantification of myogenic transcripts, qRT-PCR analysis was performed using the iCycler iQ Real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and EvaGreen (Biotium, Fremont, CA, USA). The PCR conditions were 94℃ for 5 min, followed by 35 cycles at 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 30 s. Melting curve profiles were analyzed for the amplicons. Quantitative real-time PCR (qRT-PCR) data of the target genes were normalized relative to glyceraldehyde 3-phosphate dehydrogenase expression and calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Western blotting

Total protein was extracted with 1× radioimmunoprecipitation lysis buffer and separated on a 10% polyacrylamide gel followed by transfer to a nitrocellulose membrane (Bio-Rad, USA). The primary antibodies used were mouse anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA), anti-MyoD (Santa Cruz Biotechnology, USA), anti-desmin (Novus Biologicals, Littleton, CO, USA), and anti-MF20 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA). HRP-conjugated anti-mouse IgG or anti-rabbit IgG (Bio-Rad, USA) were used as secondary antibodies. The blots were treated with enhanced chemiluminescence substrate solutions and exposed in a ChemiDoc XRS System (Bio-Rad, USA) to detect chemiluminescence.

3. Results

3.1. CRISPR/Cas9-mediated MyoD knockout and establishment of single cell-derived subline

3.1.1. MyoD gene knockout in QM7 cells.

We have to find out the genomic structure of the quail MyoD gene. So we designed the RT-PCR primers for target site from chicken MyoD gene sequences and performed sequencing. Based on the sequence information that we confirmed, we designed the target guide RNA (gRNA) site followed by a protospacer adjacent motif sequence (Figure 2).

Figure 2. The genomic sequence and structure of the quail MyoD gene.

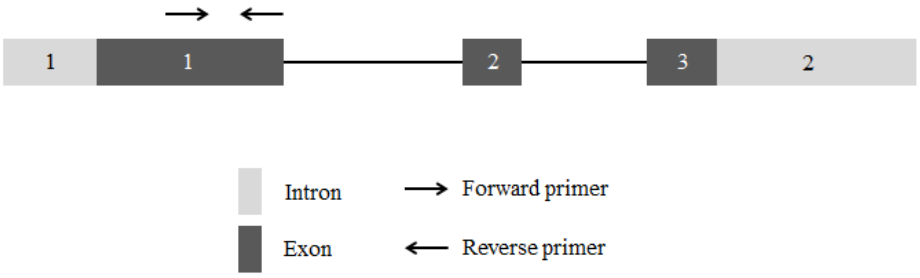

Figure 3. The genomic structure of the quail MyoD gene.

We designed and constructed an all-in-one vector. It can express both the MyoD gRNA regulated by the U6 promoter and Cas9 controlled by the CMV promoter simultaneously (Figure 4). The U6 promoter controls MyoD gRNA transcription followed by a termination signal.

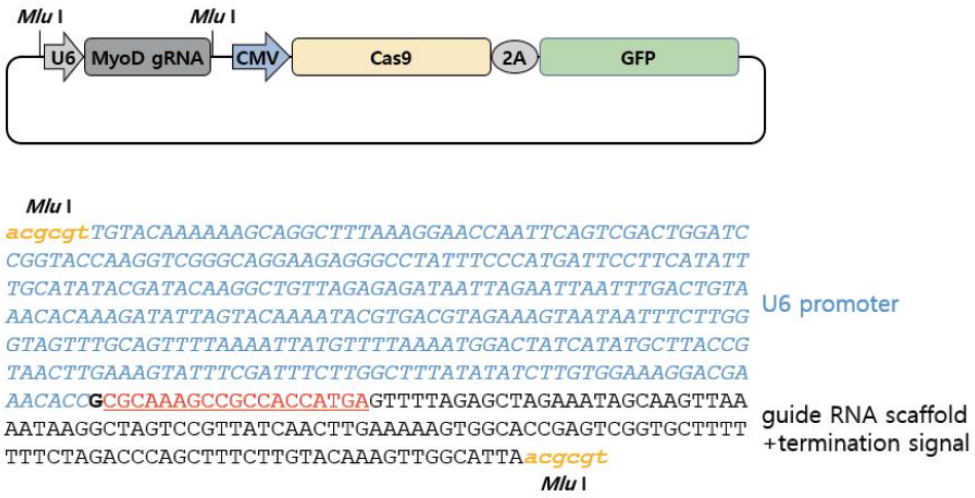

Figure 4. All-in-one vector that express both Cas9-GFP and MyoD gRNA.

After transfection of the Cas9-MyoD gRNA expression vector into QM7 cells, we sorted only GFP-positive cells by using FACS (Fluorescence activated cell sorter). And finally, a single cell was isolated from the mixed population and we expanded it *in vitro* (Figure 5).

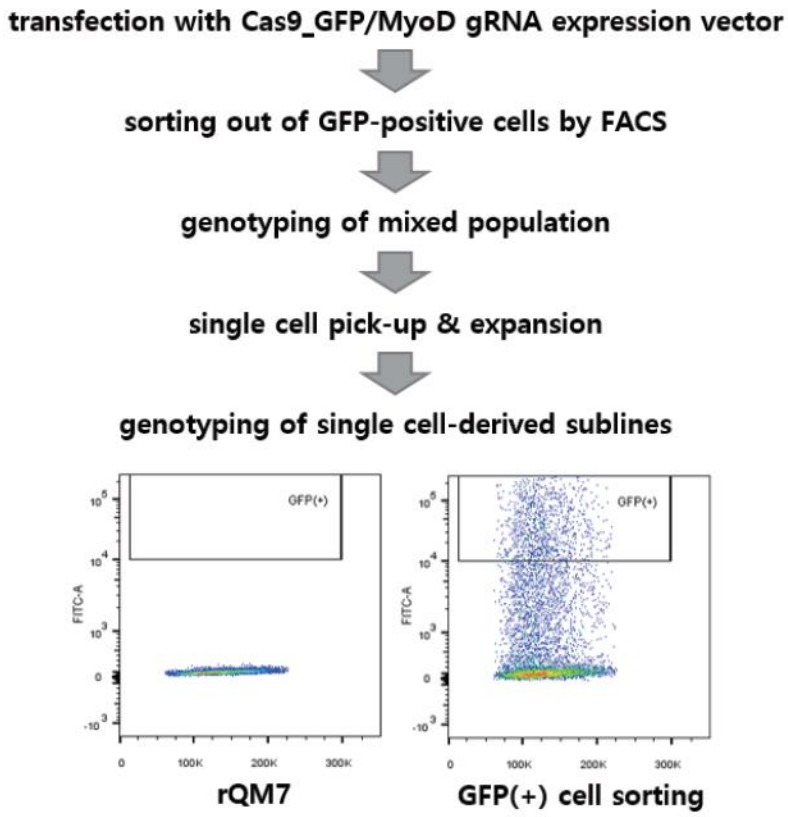

Figure 5. Overview of our experiment and fluorescence-activated cell sorting (FACS) for the identification of single cell-derived MyoD knockout QM7 sublines.

3.1.2. Genotyping analysis of MyoD Knockout QM7 cell line.

We identified some different mutant genotypes of the mixed population of QM7 cells. The mutation rate was 73.3%. 11 cells of 25 samples were mutated. We were able to see the 1~28-nucleotide deletion form, and also two different type of 1-nucleotide insertion form (Figure 6).

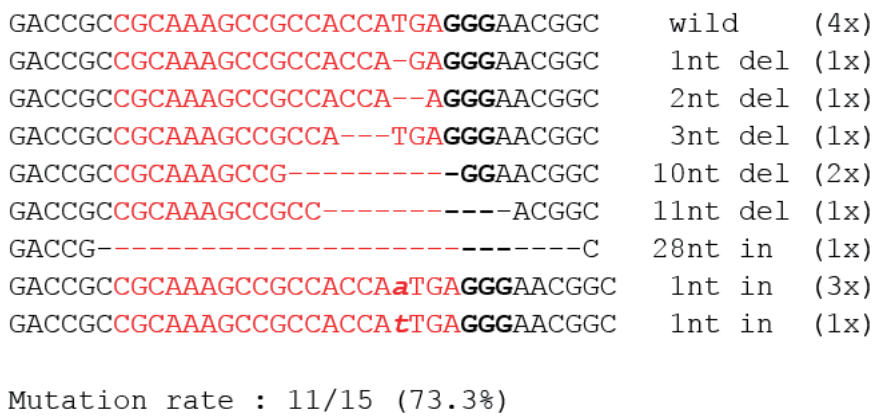

Figure 6. The diverse mutation genotypes of the mixed QM7 cell population. The number of each type of cells are shown in parenthesis on the right.

And next, after confirmed several kinds of mutation was revealed, we excluded sublines which had insertion or deletion of multiple of three nucleotides to the find frameshifted ones. Multiple of three nucleotides don't matter to function because those are moving as one amino acid. We selected a single cell-derived subline and named MyoD KO QM7#4. It has 1-nucleotide deletion type and 1-nucleotide insertion type (Figure 7). We used this MyoD KO QM7#4 cell line with single nucleotide deletion and insertion for our experiment.

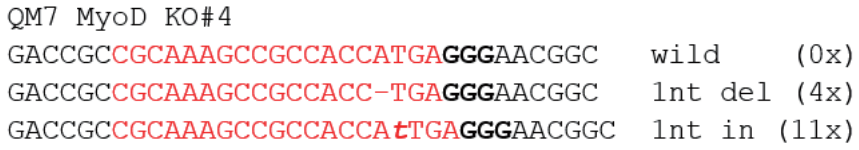

Figure 7. Genotypes of single cell-derived MyoD knockout QM7 cell subline, with single nucelotide deletion and insertion form, used for our following experiment. The number of each type of cells are shown in parenthesis on the right.

3.2. Characterization of MyoD Knockout QM7 cell.

3.2.1. Paired box 7 (Pax7) expression in rQM7 cells and MyoD KO QM7#4 cells.

Using qRT-PCR, we compared the expression of paired box 7 (Pax7) in regular QM7 (rQM7) and MyoD KO QM7#4 cells in the undifferentiated state (Figure 8, 9). Pax7 is globally well-known gene as a marker of undifferentiated myoblasts. There were no significant differences between rQM7 and MyoD KO QM7#4 cells (Figure 8, 9).

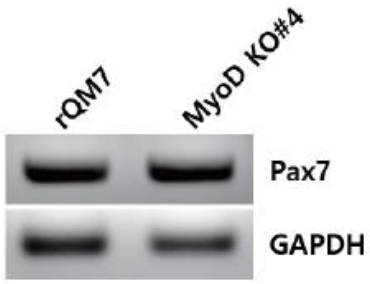

Figure 8. The data of the expression of Pax7 in rQM7 and MyoD KO QM7#4 cells by qRT-PCR.

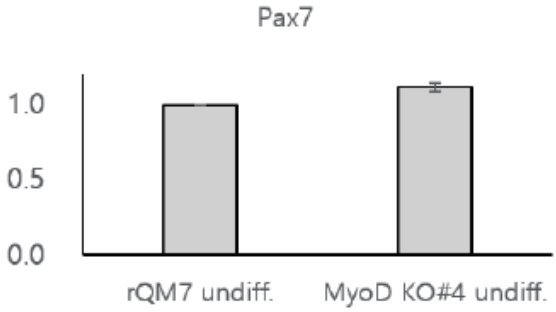

Figure 9. The quantitative data of Figure 8.

3.2.2. Morphological comparison between undifferentiated rQM7 cells and MyoD KO QM7#4 cells.

There were no significant differences also in the morphology of the rQM7 and MyoD KO QM7#4 cells in undifferentiated state. (Figure 10).

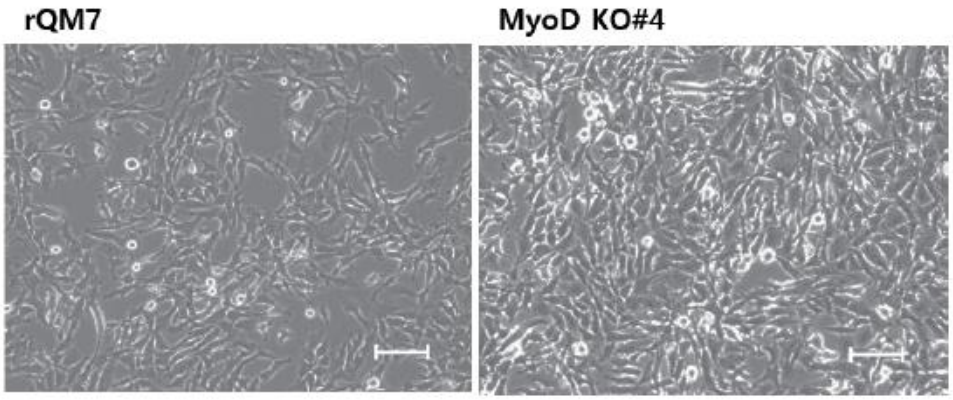

Figure 10. The morphology of the rQM7 and MyoD KO QM7#4 in undifferentiated state. There were no significant differences between two of them. The images were observed under the inverted microscope, scale bars = 200㎛.

3.2.3. Morphological comparison between differentiated rQM7 cells and MyoD KO QM7#4 cells.

When we converted the medium into a differentiation condition, rQM7 cells were differentiated into myotubes. Changes began in the 3 days after the differentiation. We can see the transition to myotube formation obviously on 3 and 6 days in rQM7 cells (Figure 11-A, B). But MyoD KO QM7#4 cells did not show any visible changes under the differentiation condition despite 6 days after differentiation (Figure 11-A, B).

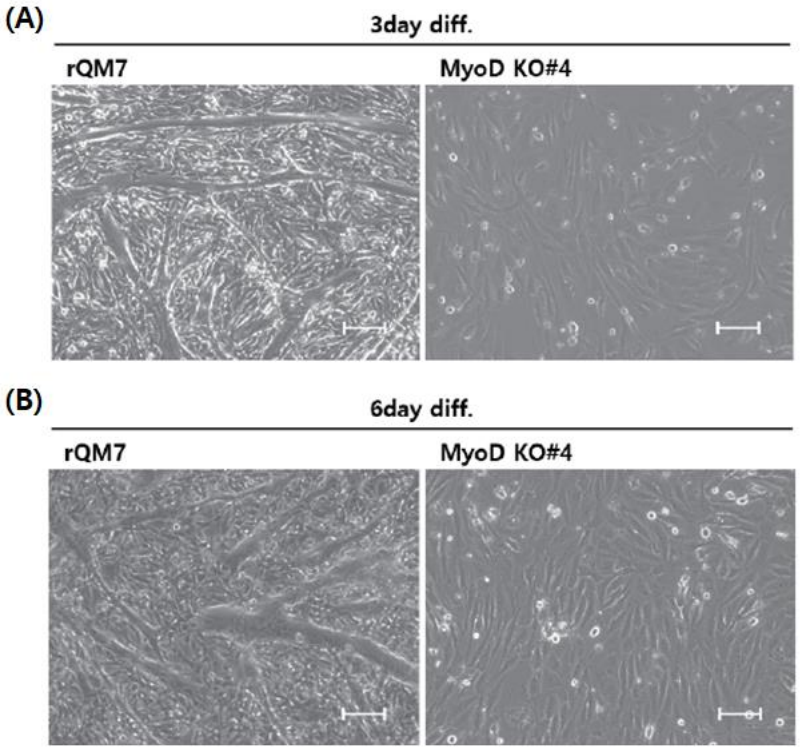

Figure 11. Morphological changes under differentiation condition. We can see the difference clearly after differentiation as rQM7 cells, whereas MyoD KO QM7#4 cells were not at all likely to. The images were observed under the inverted microscope, scale bars = 200㎛.

3.2.4. Nuclear fusion of the rQM7 cells and MyoD KO QM7#4 cells.

We did DAPI (4', 6-diamidino-2-phenylindol dihydrochloride) staining to confirm both the myotube formation and nuclear fusion of the rQM7 cells and MyoD KO QM7#4 cells on the 3, 6 day after differentiation. There were many fused nuclei in the myotubes of the differentiated rQM7 cells (Figure 12-A). However, we cannot found any myotube formations or fused nuclei in MyoD KO QM7#4 cells during differentiation (Figure 12-B, C).

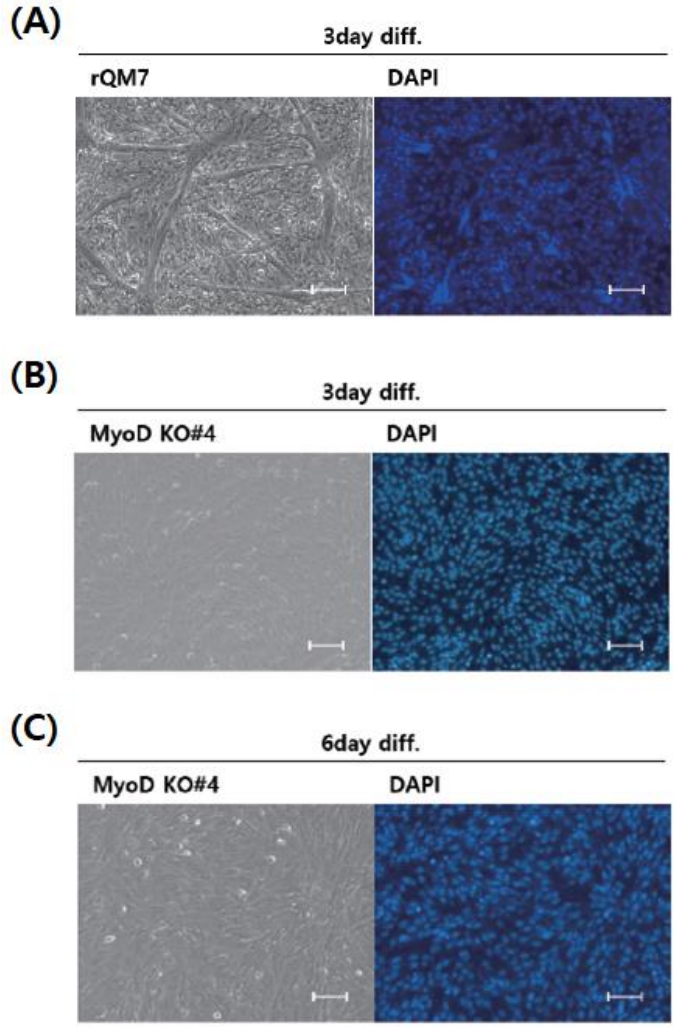

Figure 12. DAPI (4', 6-diamidino-2-phenylindol dihydrochloride) staining after differentiation. The images were observed under the inverted microscope, scale bars = 200㎛.

3.2.5. Confirm the expression of myogenic differentiation-related genes in rQM7 cells and MyoD KO QM7#4 cells.

To check the expression of MyoD protein, we did Western blot analysis 3 day of differentiation. As compared with rQM7, MyoD proteins were not detected in MyoD KO QM7#4 cells (Figure 13).

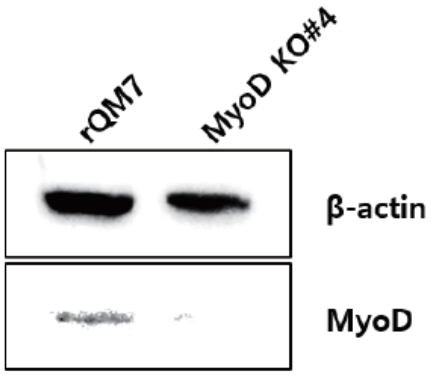

Figure 13. Western blot assay of functional MyoD genes.

We analyzed myogenic differentiation-related genes in 3, 6 days of differentiation by RT-PCR to confirm myogenic gene induction on the differentiation condition. MyoD expression in rQM7 and MyoD KO QM7#4 cells were both increased as date passes after differentiation. And we found big gaps of MyoD expression in rQM7 cells on 6 days differentiation (Figure 14-A, B). And we also found that the myogenic transcripts like Myogenin and Desmin were not detected both on 3 and 6 days of the differentiation (Figure 14-A, B).

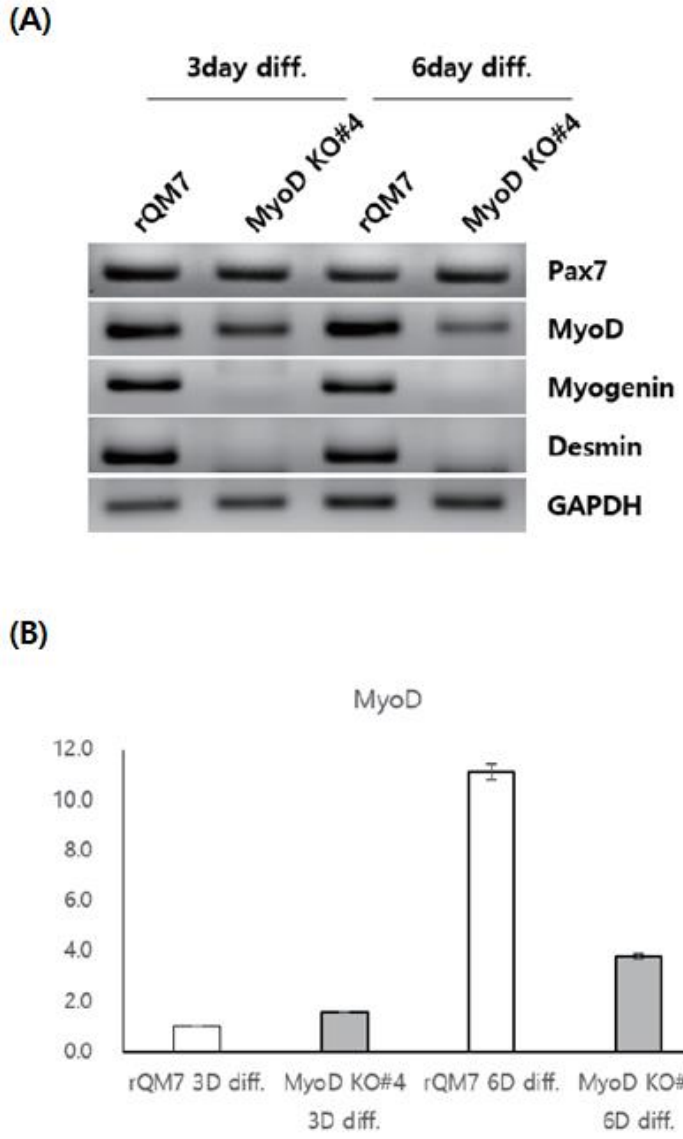

Figure 14. (A) RT-PCR analysis of myogenic differentiation-related gene. (B) Quantitative RT-PCR analysis of MyoD gene in rQM7 cells and MyoD KO QM7#4 cells during differentiation.

To confirm myogenic transcripts during differentiation, we performed Western blot analysis. Myogenic terminal proteins, including Desmin and Myosin Heavy Chain (MF20), were not detected in the differentiated MyoD KO QM7#4 cells but were detected in the differentiated rQM7 cells (Figure 15).

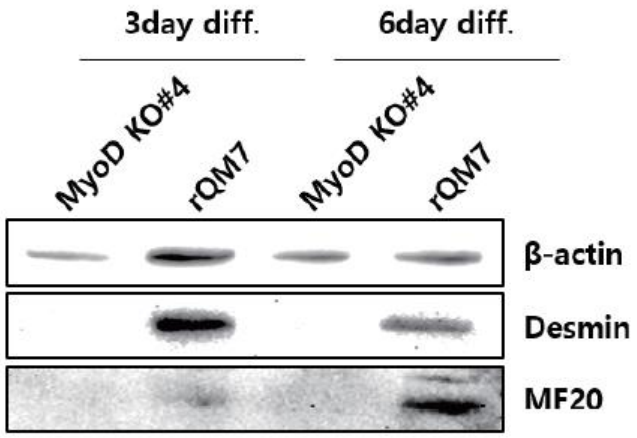

Figure 15. Western blotting analysis of myogenic terminal proteins (Desmin and MF20) during differentiation.

3.3. Global gene expression analysis by RNA sequencing in QM7 MyoD KO cells

There is no formal genome information on quail, so we had to perform *de novo* assembly by RNA-sequencing both of the differentiated rQM7 and MyoD KO QM7#4 sublines. The string analysis of differentially expressed genes (DEGs) of rQM7 cell and MyoD KO QM7#4 cells by RNA-sequencing data is shown in figure 15. DEGs in figure 16 which were significantly decreased in MyoD KO QM7#4 cells. The global myogenic genes were greatly downregulated in MyoD KO QM7#4 cells during differentiation and their myogenic regulatory pathway was also suppressed in MyoD KO QM7#4 cells (Figure 16, 17). And we organized the top 30 genes that downregulated in the differentiated MyoD KO QM7#4 cells, such as Troponin T (TNNT2), Myosin regulatory light chain 2B (MLRB) and Myogenin, to Table 2 in the lower section.

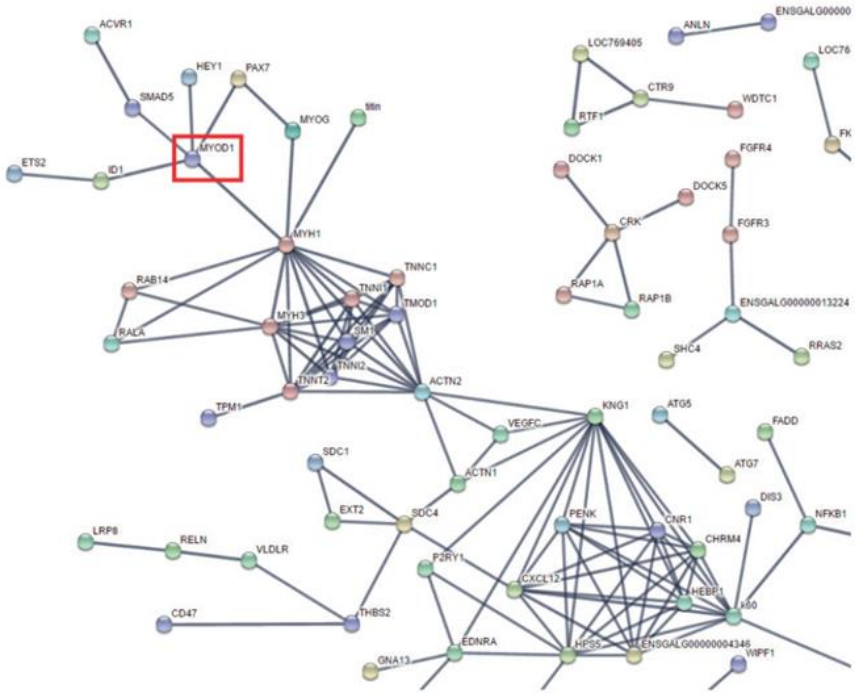

Figure 16. String analysis of total differentially expressed genes (DEGs) processed by RNA-sequencing data of rQM7 and MyoD KO QM7#4 cells during differentiation. The red box is the MyoD transcript.

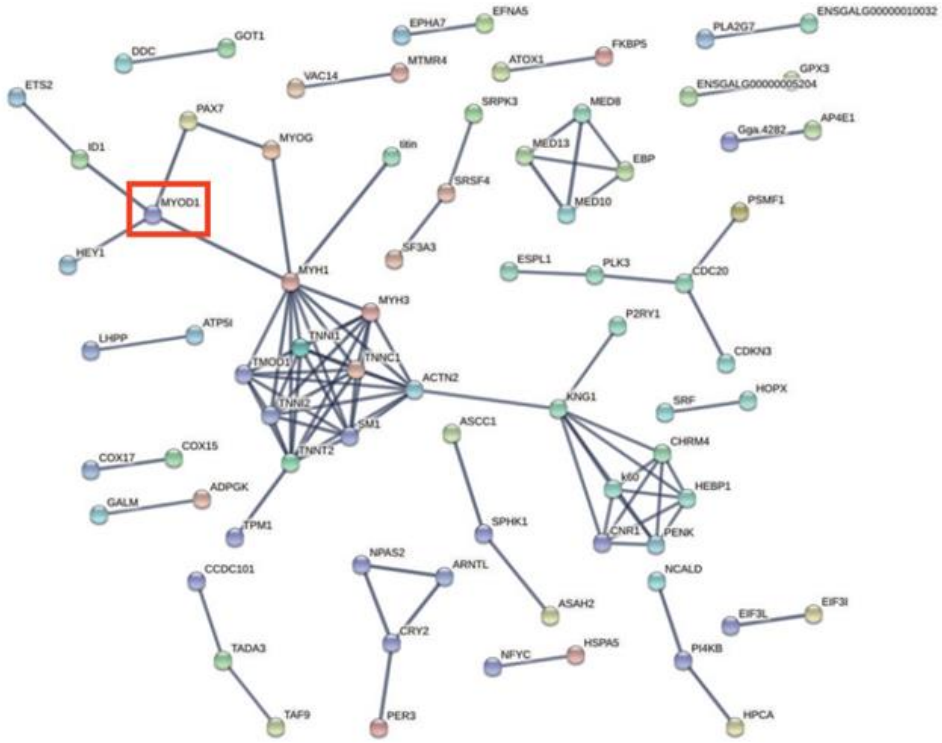

Figure 17. String analysis of differentially expressed genes (DEGs) that significantly decreased in MyoD KO QM7#4 cells during differentiation. The red box is the MyoD transcript.

Table 2. Top 30 genes down-regulated in the differentiated MyoD KO QM7#4 cells compared to the rQM7 cells.

Gene	Description	Fold change (Log ratio)	FDR	P-Value
TNNT2	Troponon T, cardiac muscle isoforms	-17.4203	0	0
MLRB	Myosin reglatory light chain 2B, cardiac muscle isoform	-17.3022	0	0
MYOG	Myogenin	-15.9997	0	0
MYBPH	Myosin-binding protein H	-15.6882	0	0
CASQ2	Calsequestrin-2	-15.1500	0	0
ACHG	Acetylcholine receptor subunit gamma	-14.8214	0	0
MLE1	Myosin light chain 1, skeletal muscle isoform	-14.6863	0	0
DESM	Desmin	-14.2803	0	0
CAV3	Caveolin-3	-14.2394	0	0
EF1A2	Elongation factor 1-alpha 2	-13.9406	0	0
TNNI1	Troponin I, slow skeletal muscle	-13.8945	0	0
DUS26	Dual specificity protein phosphatase 26	-13.8884	0	0
MLEC	Myosin light chain 1, cardiac muscle	-13.5580	0	0
KCRM	Creatine kinase M-type	-13.3336	1.72E-266	6.84E-270
DDR1	Epithelial discoidin doain-containing receptor 1	-13.2175	3.55E-231	1.74E-234
MYOZ2	Myozenin-2	-12.8902	2.29E-202	1.33E-205
TNNT2	Troponin T, cardiac muscle	-12.8211	9.12E-191	5.66E-194
MLE3	Myosin light chain 3, skeletal muscle isoform	-12.7211	1.10E-181	7.49E-185
TNNI2	Troponin I, fast skeletal muscle	-12.6837	0	0

TBA3	**Tubulin alpha-3 chain**	**-12.5555**	**0**	**0**
POPD1	**Blood vessel epicardial substance**	**-12.5325**	**2.81E-161**	**2.29E-164**
LOLA1	**Longitudinals lacking protein, isoform G**	**-12.4171**	**1.01E-147**	**9.11E-151**
TBA3	**Tubulin alpha-3 chain**	**-12.3884**	**0**	**0**
TBA1C	**Tubulin alpha-1C chain**	**-12.1987**	**0**	**0**
HSPB2	**Heat shock protein beta-2**	**-12.1131**	**0**	**0**
TM182	**Transmembrane protein 182**	**-11.8558**	**2.24E-99**	**3.30E-102**
SH3BG	**SH3 domain-binding glutamic acid-rich protein**	**-11.8005**	**1.67E-95**	**2.60E-98**
MNME	**tRNA modification GTPase**	**-11.7358**	**8.87E-89**	**1.55E-91**
MYH6	**Myosin-6**	**-11.5455**	**0**	**0**
MALQ	**4-alpha-glucanotransferase**	**-11.5129**	**2.55E-75**	**5.43E-78**

4. Discussion

The CRISPR/Cas9 system is efficient tool for genomic modification. It has being utilized for diverse applications in the scientific study (Cong, Ran et al. 2013, Mali, Yang et al. 2013). This technique has greatly developed bioscience and technology with its simple and high efficiency of genomic editing (Jao, Wente et al. 2013, Wang, Yang et al. 2013, Hai, Teng et al. 2014). Unlike ZFN(1^{st}) and TALENs(2^{nd}) have been used to be genomic editing before (Gaj, Gersbach et al. 2013, Park, Lee et al. 2014), CRISPR/Cas9 system is more efficient for large-scale and multigene-editing in many species, particularly avian species.

We knocked out a specific myogenic regulation-related gene and tried to figure a regulatory pathway out. It could be used to advance quality like growth in the poultry industry.

The MyoD KO QM7#4 subline, with single nucleotide insertion and deletion, was used to investigate some phenomenon in this study (Figure 7). The expression of Pax7 was not significant between rQM7 cells and the MyoD KO QM7#4 cells (Figure 8, 9). Pax7 is one of the global marker of undifferentiated myoblasts, so the data suggested that deletion of MyoD gene did not impact on myogenic mechanism under undifferentiation condition. Since the differentiation was induced, MyoD KO QM7#4 cells did not change into myotubes (Figure 11). It was a strikingly different from the result of the differentiated rQM7 cells. The differentiated rQM7 cells were transformed into myotube, and many fusioned nucleus were identified in the differentiated rQM7 cells (Figure 12). Although functional MyoD was lack in MyoD-knockout mice, the expression of them are still viable (Rudnicki, Braun et al. 1992,

Arnold and Braun 2003). They also do not exhibit any abnormal behaviors in skeletal muscle (Rudnicki, Braun et al. 1992, Arnold and Braun 2003).

We could identify several critical myogenic regulatory-related genes, which expressions were decreased in MyoD KO QM7#4 cells compared to rQM7 cells, through RNA sequencing analysis (Figure 16, 17, Table 2). Some of them are paired box 7 (Pax7), myosin heavy chain 1 (MYH1), myogenin (MyoG), hairy/enhancer-of-split related with YRPW motif protein 1 (Hey1), activing A receptor type 1 (ACVR1), Troponin T (TNNT), myosin regulatory light chain 2B (MLRB), SH3 domain-binding proteins, and so on (Figure 16, 17, Table 2). Some of those genes including Pax7, Id1, MyoG, Hey1, ETS2, turned out that associated with MyoD in the second DEG analysis data which were downregulated in MyoD KO cells compared to rQM7 cells. In fact, studies on the interaction between these genes and MyoD gene is proceeding relatively strong. Pax7, plays major roles during fetal development, is essential for renewal and maintenance of muscle cells. Particularly, expression of Pax7 is well-known as being closely related with MRFs, including MyoD and Myf5, and their interaction is important in proliferation and differentiation in skeletal muscle(Ropka-Molik, Eckert et al. 2011). MyoD gene regulates differentiation with Pax7 in activated satellite cells of the muscle cells (Mesires and Doumit 2002). And also, repression of myogenesis via inhibition of MyoD activity by Pax7 is revealed from other study (Olguin, Yang et al. 2007). According to widely known general outline, some other genes, such as MRF4 and Pax3, are involved in the pathway from determination to differentiation of muscle specific proteins.

Investigating the detailed mechanisms of their interaction might be essential to improve avian traits to apply in the livestock industry. In addition, since the pathways might be different among species, we have to study about myogenic regulatory pathway of avian species compared to other species including mammal. And also, genes which are not connected with MyoD or are located far from MyoD in the figure may have no interaction with MyoD. However, it is also possible that there have been any investigation on such interaction at all. Therefore, it is required to study interaction between MyoD and other genes.

There was a study about transition, a nestin-like intermediate filament protein, on the impact on the expression of MyoD, myogenin and pax7. There was a study about transition, a nestin-like intermediate filament protein, on the impact on the expression of MyoD, myogenin and pax7 (Jalouli, Lapierre et al. 2010). Insulin and insulin signaling(insulin/PI3K/Akt) pathways are related in the process of myogenesis at the differentiation stage in chicken myoblasts on the other report (Sato, Aoki et al. 2012). MYOG and MRF4 are expressed late embryo development. MYOG is related to the determination of myoblast differentiation, and Mrf4 is expressed in mature myocytes (Nabeshima, Hanaoka et al. 1993, Stern, Ashwell et al. 2015). PAX7 expressed in proliferating myoblasts as a transcript factor, but it was downregulated during myoblast differentiation (Seale, Sabourin et al. 2000, Zammit, Golding et al. 2004, Stern, Ashwell et al. 2015). In the other study, they confirmed that the expression of Pax7 and Myf5 decreased in the Sema3A siRNA-transfected myoblasts. However, these cells did not activate the expression of MyoD during differentiation (Qahar,

Takuma et al. 2016). Many research on the relevance between MyoD and ID1 in the ubiquitin pathway are ongoing (Hatoum, Gross-Mesilaty et al. 1998, Trausch-Azar, Lingbeck et al. 2004). Also Notch signaling pathways, which regulate MRFs and Hey1 gene in skeletal myogenesis, are actively being studied (Buas, Kabak et al. 2009, Buas, Kabak et al. 2010, Buas and Kadesch 2010). Now in my laboratory, we were processed a lot of studies on relevance between MyoD and Pax7 of the most, but there are so many things to discover through several studies on the pathways in myogenesis.

Finally, we successfully deleted MyoD gene by CRISPR/Cas9 mediated knockout technique in avian myoblasts. With CRISPR/Cas9-mediated genomic editing, single cell-derived sublines with a specific knockout gene could be adapted to various aspects of basic research for functional genomics studies of mammal, as well as practical application for the avian industry.

5. Conclusion

Utilizing the single cell-derived MyoD gene knockout sublines by CRISPR/Cas9-mediated genomic editing, we found significant differences between regular QM7 (rQM7) and MyoD KO QM7#4 cells during differentiation. rQM7 changed into myotube since 3 days after differentiation. However, there were no changes in the differentiated MyoD KO QM7#4 cells were observed even 6 days after differentiation. We confirmed the phenomenon by diverse experiment, such as 4', 6-diamidino-2-phenylindol dihydrochloride (DAPI) staining, RT-PCR, quantitative RT-PCR, and Western blot analysis. Consequently, the results of this study might be concluded that MyoD plays an important role in muscle differentiation. In addition to the results, this experiment also suggests that the CRISPR/Cas9 system would be useful genomic editing for the poultry genome sciences in the future.

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7. 요 약

축산 분야에서 가축의 성장과 축산물 생산성의 향상에 대한 연구는 매우 중요하다. 그 중에서도 가축의 골격근의 성장을 연구하는데 있어서 근육세포의 증식과 분화를 조절하는 메커니즘을 알아내는 것은 꼭 필요하다. 이것은 대동물의 경우에만 국한되는 것이 아니라 조류의 경우에도 마찬가지이다. 그래서 본 연구에서는 메추리의 근원세포인 QM7 cell line 을 이용해서, 근육분화에 큰 연관이 있다고 알려져 있는 MyoD 의 조절 경로와 MyoD 가 근육분화에 미치는 영향에 대해 알아보고자 했다. Myoblast 가 증식 이후 초기 myotube 로 분화하는 과정에서 MyoD 의 발현이 줄어들면 myotube 로 분화하는데 영향이 있을 것이라 가정하고, CRISPR/Cas9 시스템을 이용해서 메추리 근원세포에서 MyoD 를 knockout(KO) 시킨 후에 발생하는 변화를 확인해서 myotube 부화 과정에서 MyoD 가 어떤 영향을 주는지 알아보고자 한 것이다. 본 연구에서 진행된 실험에는 교정을 하지 않은 rQM7 cell 과 유전자 교정 기술 (CRISPR/Cas9)을 이용해서 MyoD 유전자를 knockout 시킨 mutant subline 이 이용되었다. 미분화 단계에서 rQM7 과 MyoD KO QM7 cell 에서 Pax7 이라는 myoblast 미분화 마커의 발현과 형태는 유의적인 차이가 없었다. 분화를 진행한 이후 3, 6 일째에 rQM7 과 MyoD KO QM7 cell 을 비교한 결과, rQM7 은 3 일째에 myotube 로 분화가 진행되었으나 MyoD KO QM7 cell 은 그렇지 않음을 확인할 수 있었다. DAPI 염색을 실시해서 핵 융합을 확인했을 때도 같은 결과를 보였다.

분화가 진행된 이후의 rQM7 의 myotube 에서는 핵이 융합된 형태가 많이 발견되었는데, MyoD KO QM7 cell 에서는 분화 후 6 일째에도 분화된 myotube 나 융합된 핵을 발견할 수 없었다. 또한 Western Blotting 을 통해 근분화-특이 transcript 와 단백질들이 MyoD KO QM7 cell 에서는 발현되지 않는 것도 확인하였다. 따라서, 본 연구의 결과는 MyoD 가 근육분화에 중요한 역할을 하는 요인이라는 결론으로 도출될 수 있으며, CRISPR/Cas9 시스템을 이용한 조류의 유전자교정이 가능하다는 것을 다시 한번 검증함으로써 차후 이를 이용한 유전적 연구가 활발히 이루어질 수 있다는 점을 시사한다고 판단된다.

주요어: 근원세포, CRISPR-Cas9, 녹아웃, 근육분화, MyoD

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