Master's Thesis of Science in Agriculture

Functional Analysis of Forkhead Box O3 (FOXO3) During the Myogenic Differentiation in Chicken Myoblast Cells

닭의 근아 세포의 근육 분화 과정에서

Forkhead Box O3 (FOXO3)의 기능 분석

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Functional Analysis of Forkhead Box O3 (FOXO3) During the Myogenic Differentiation in Chicken Myoblast Cells

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Abstract

In poultry industry, growth performance of chicken is important in that it affects to economic values in many ways. Investigations of specific genes and proteins which are regulators of muscle proliferation and differentiation were introduced to selective-breeding approaches. We conducted the functional analysis of *Forkhead Box O3 (FOXO3)* during the myogenic differentiation in chicken myoblast cells. We established pCM cell lines which were derived from pectoralis of 10 days-old male Hy-Line Brown via primary culture. The pCM cell lines were stable and immortal chicken myoblast cells. The *FOXO3* gene was down-regulated by applying RNA interference molecules which consisted of two miRNAs and a *piggyBac* transposon vector. Thus, Foxo3 knockdown cell lines were identified to analyze the functions and effects, responsible for muscle differentiation. The pCM Foxo3 knockdown cell line showed significantly down-regulated the Foxo3 expression. In the undifferentiated state, the down-regulated Foxo3 decrease expression of other genes related with growth and differentiation of chicken myoblasts. Moreover, in the differentiated state, expression of Foxo3, MyoD, Pax7, CEBPB and SRF was down-regulated in the pCM Foxo3 KD cells. However, the differentiation marker, Desmin and Myogenin

expressed at the high level. Furthermore, down-regulated Foxo3 made the chicken myoblast cells grow slowly, differentiate rapidly in induced differentiation and form unusual myotubes, which had thinner and longer and lower number of nuclei in differentiated area than regular pCM cells. These results demonstrated that Foxo3 is related with growth and differentiation of chicken myogenic cell lines via interactions with other transcription factors. Therefore, the Foxo3 can be applied to selective-breeding approaches in poultry industry.

Keyword: Foxo3, miRNA, knockdown, muscle differentiation, chicken myoblast cell line

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List of Abbreviation

FOXO3 : Forkhead Box O 3

MyoD : Myogenic Differentiation

Pax7 : Paired Box 7

CEBPB : CCAAT/Enhancer Binding Protein β

SRF : Serum Response Factor

SRE : Serum response element

qRT-PCR : Quantitative Real time-Polymerase Chain Reaction

rpCM : Regular pCM

KD : Knockdown

DNA : Deoxyribonucleic Acid

cDNA : complemntary DNA

RNA : Ribonucleic Acid

RNAi : RNA interference

miRNA : micro RNA

siRNA : small interfering RNA

mRNA : messenger RNA

dsRNA : double-stranded RNA

RISC : RNA-induced silencing complex

MRFs : Myogenic Regulatory Factors

PKB : Protein kinase B

PI3K : Phosphatidlyinositol 3-kinase

Bim : Bcl-2-like Protein 11

PUMA : p53 upregulated modulator of apoptosis

FLIP : FLICE-like inhibitory protein

bHLH : Basic helix-loop-helix

IL-4 : Interleukin-4

IL-6 : Interleukin-6

CMV : Cytomegalovirus

EF1 : Elongation factor 1

FBS : Fetal bovine serum

PBS : Phosphate-buffered saline

RIPA : Radioimmunoprecipitation

ECL : Enhanced chemiluminescence

SAS : Statistical analysis system

DAPI : 4',6-diamidino-2-phenylindole

Hes1 : Hes family bHLH transcription factor 1

Hes2 : Hes family bHLH transcription factor 2

Hes6 : Hes family bHLH transcription factor 6

HeyL : Hes related family bHLH transcription Factor with YTPW motif-like

Chapter 1. Introduction

Chicken growth performance is determined by environmental, nutritional and genetic factors. It is important as it is related to economic advantages of the poultry industry (Claire D'Andre et al., 2013). Thus, investigation of specific genes and proteins is important because these genes are used for selective-breeding approaches (2004). Selective-breeding approaches have been used in the poultry industry for a long time in other for generating specialized commercial populations (Rubin et al., 2010). Since whole genome sequences including avian species are publicly revealed, a mass of information has been provided for functional genomics.

Functional genomics has been widely used because it is one of effective ways to understand the growth of skeletal muscles, such as

the mechanisms of muscle proliferation and differentiation (Al-Musawi et al., 2011, Li et al., 2011, Shin et al., 2015). Especially, the genetically engineered livestock can rapidly improve on economic traits such as growth performance and disease resistance. There are two strategies of functional genomics analysis, which are acquisition and loss of functions. Former the function is strategy that delivers and expresses foreign genes by using expression promoters and vector system. Latter one is to make their functions lose partially or fully in the gene products (Muller, 1932). In this study, we use the latter, loss of function strategy, by using miRNA, which is transferred by *piggyBac* transposon vector system.

miRNA is a type of small RNA molecules which is used to interfere target molecules. This RNA interference (RNAi) is an efficient knockdown procedure by modifying target genes (Fire et al., 1998).

miRNA 126 based design can express one to three miRNAs from single miRNA 126 based cassette and miRNAs form hairpin structures for knockdown specific genes (Chen et al., 2011). However, in order to knockdown target genes, the gene delivery system is required because miRNA molecules should be transferred into the host cells. We use *piggyBac* vectors to transfer miRNAs. Two miRNAs are inserted into the *piggyBac* vectors, and these vectors are transfected by transposase into the host cells.

Previous studies demonstrated highly efficient gene delivery systems in chicken by using *piggyBac* transposon and transposase, which produces transgenic chickens with the stable transgene expression without any tissue-specific repression (Park and Han, 2012b, Love et al., 1994, van de Lavoir et al., 2006). Because this system is non-viral production system, there is no biosafety issue of

virus mediated system (Salter et al., 1986). Therefore, *piggyBac* system is expected to be a practical, industrial liable applications (Macdonald et al., 2012).

These genetically modified systems in organisms including avian species make a model organism to study various diseases and develop the pharmaceutical drugs (Park and Han, 2012a). In addition, as genome editing has developed to be accurate and pragmatic, interest in utilization of genome editing in agricultural industry has been increased.

Chapter 2. Review of Literature

1. Growth and differentiation of chicken myoblast

1) Growth and differentiation of myoblast

To form myofiber, myogenic precursors must be activated at the first stage. Myogenic precursors known as satellite cells are required postnatal growth and regeneration of skeletal muscle (Olguin et al., 2007). Satellite cells located on the sarcolemma of muscle fiber are able to differentiate and have potential to form additional myofiber as a response to injury or disease (Zammit et al., 2006). In general, most of satellite cells, undamaged muscle are in the quiescent stage, which neither proliferate nor differentiate (Birbrair and Delbono,

2015).

In response to stimuli such as injury or disease, satellite cells become activated and small population of satellite cells return to a quiescent state referred to self-renewal (Kadi et al., 2004, Birbrair and Delbono, 2015). When quiescent satellite cell is activated, satellite cells enter the cell cycle and grow.

Myoblasts are embryonic cells that become a cell of muscle fiber. They are proliferating satellite cells and undifferentiated muscle cells, which become myotubes through differentiation (Scott et al., 2001). After several rounds of proliferation, myoblasts enter the terminal differentiation stage. In this stage, one or several myoblasts fuse into new myotubes (Olguin and Pisconti, 2012). Finally, the myotubes form new myofibers (Figure 1).

2) pCM cell

In avian, the QM7 cell line is the only muscle myoblast cell. This cell line derived from the quail fibrosarcoma cell was isolated and characterization. This development helps understanding of muscle development and myogenic differentiation (Antin and Ordahl, 1991). Moreover, it is useful for discovering a relationship between myogenesis and cell cycle and investigating extracellular signals and muscle-specific genes which are influence muscle differentiation (Florini and Magri, 1989). However, there is no chicken myogenic cell line. Thus, we were able to achieve only limited successes.

Therefore, we isolate primary culture cells from pectoralis of 10 days-old male Hy-Line Brown (Danoviz and Yablonka-Reuveni, 2012). In general, primary culture cells are subcultured only 5 to 10 passages. However, primary culture cells we isolated were able to

continue subculturing without additional immortalize procedures. We suggest that our primary culture cells spontaneously become stable cell lines and design them as pCM cell line. In addition, pCM cell lines are chicken muscle myoblast which can differentiate via serum. Thus, the establishment of pCM cell lines provide opportunity of studies about chicken myogenesis and cell cycle.

2. *Forkhead Box O3 (FOXO3)*

1) *Forkhead Box O3 (FOXO3)*

The Forkhead box class O transcription factor family has several functions for cells such as determination of cell fate, cell growth, differentiation, DNA damage repair, apoptosis, energy metabolism, resistance to oxidative stress and longevity (Medema et al., 2000,

Tran et al., 2002, Kops et al., 2002, van der Vos and Coffer, 2011, Warr et al., 2013, Sanchez et al., 2014, Morris et al., 2015). Furthermore, the *Foxo1, Foxo2, Foxo3* null type mice indicate specific phenotypes that every *Foxo* gene has specific roles and is functionally different (Hosaka et al., 2004, Castrillon et al., 2003, Jonsson et al., 2005, Lin et al., 2004, Paik et al., 2007). Especially, the *Foxo3* null type mouse is viable and shows age-dependent infertility, unusual development of ovarian follicular, lymphoproliferation, impaired muscle regeneration and downregulation of MyoD (Hu et al., 2008, Ni et al., 2006, Nakae et al., 2008).

These transcription factors have the ability of inhibited and translocated out of the nucleus on phosphorylation by Akt/PKB in the PI3K signaling pathway (Brunet et al., 1999). This protein functions

initiation of apoptosis by up-regulation of necessary genes for cell death, such as *Bim* and *PUMA* or by down-regulation of protein prevented form apoptosis such as FLIP (Ekoff et al., 2007, Skurk et al., 2004). Furthermore, recent study demonstrates a functional requirement of Foxo3 as a regulator of Notch signaling pathway (Gopinath et al., 2014). Notch signaling pathway is an essential regulator of quiescent satellite cells in the self-renewal of satellite cells during muscle regeneration (Mourikis et al., 2012, Bjornson et al., 2012, Wen et al., 2012).

Because *Forkhead Box O3 (FOXO3)* is one of the Forkhead box O transcription factor family, *FOXO3* plays a critical role in many species, of course, in chicken. In this study, we focused on the function of *FOXO3* which influence on cell growth and differentiation.

2) *FOXO3* influence chicken growth

In previous studies, the functions of *Foxo* genes have been described widely in muscle growth and differentiation (Bois and Grosveld, 2003, Hribal et al., 2003, Kitamura et al., 2007, Machida et al., 2003, Sandri et al., 2004). One of them, the Foxo3, is expressed in most cell types (Anderson et al., 1998, Biggs et al., 2001). However, in muscle cells, the Foxo3 is also one of the factors that are considered to influence chicken growth.

MyoD, Pax7, CEBPB, SRF and Foxo3 seems to be crucial factors in the chicken myogenesis through different ways and interactions (Marchildon et al., 2012, Lagirand-Cantaloube et al., 2009). In this study, we investigated influence of knockdown of Foxo3 on other genes.

MyoD is the first myogenic regulatory protein to be identified which

belongs one of the earliest myogenic regulatory factors (MRFs) well known as a key factor of myogenesis (Blum et al., 2012, Davis et al., 1987). MRFs are basic helix-loop-helix (bHLH) transcription factors which are sequential in myogenic differentiation(Rudnicki et al., 1993). MyoD acts as the earliest myogenic regulatory factors for the determination and differentiation of skeletal muscle (Berkes and Tapscott, 2005). MyoD is expressed at low levels in quiescent muscle satellite cells but when muscle are responded to exercise or damage, expression of MyoD increases in myoblast in order to determine terminal differentiation (Buckingham, 2006). According to recent studies, FOXO3 directly activates MyoD by binding to the promoter of MyoD (Hu et al., 2008).

The Pax7 is a critical marker of undifferentiated myoblasts. It is implicated in satellite cell specification, self-renewal (Seale et al.,

2000, Olguin and Olwin, 2004, Oustanina et al., 2004, Zammit et al., 2004, Shinin et al., 2006). The satellite cells are quiescent myogenic progenitors in the adult skeletal muscle which is activated to regenerate in response to stimuli such as muscle injury or chronic disease (Hawke and Garry, 2001, Schultz and McCormick, 1994, Smith et al., 1994). The ratios of Pax7 and MyoD (Pax7/MyoD) are high in satellite cell stages. Moreover, when satellite cell is activated the ratios of Pax7/MyoD become lower until the terminal differentiation stage (Figure 1).

The CCAAT/enhancer binding protein beta (CEBPB) is also one of the crucial transcription factors for chicken growth that inhibits the differentiation of myoblast. Moreover, loss of CEBPB expression showed premature differentiation and even the same results were observed in high serum conditions which generally inhibits the

differentiation (Marchildon et al., 2012). Behaviors of CEBPB are similar to Pax7. They expressed at high level in the satellite cells and expressed at low levels in the differentiation stage.

Furthermore, serum response factor (SRF) is a transcription factor which is essential for an activator of serum response element (SRE) (Dalton et al., 1993). In addition, the SRF has protein-protein interaction with CEBPB to activates SRE (Hanlon and Sealy, 1999, Vialou et al., 2012). The SRF increase the muscle growth and differentiation via controlling *IL-4, IL-6* (Charvet et al., 2006, Schiaffino et al., 2013). In previous studies, RNA sequencing in QM7 cells suggested that CEBPB and SRF be associated with muscle growth and differentiation (Lee et al., 2017, Kim et al., 2017).

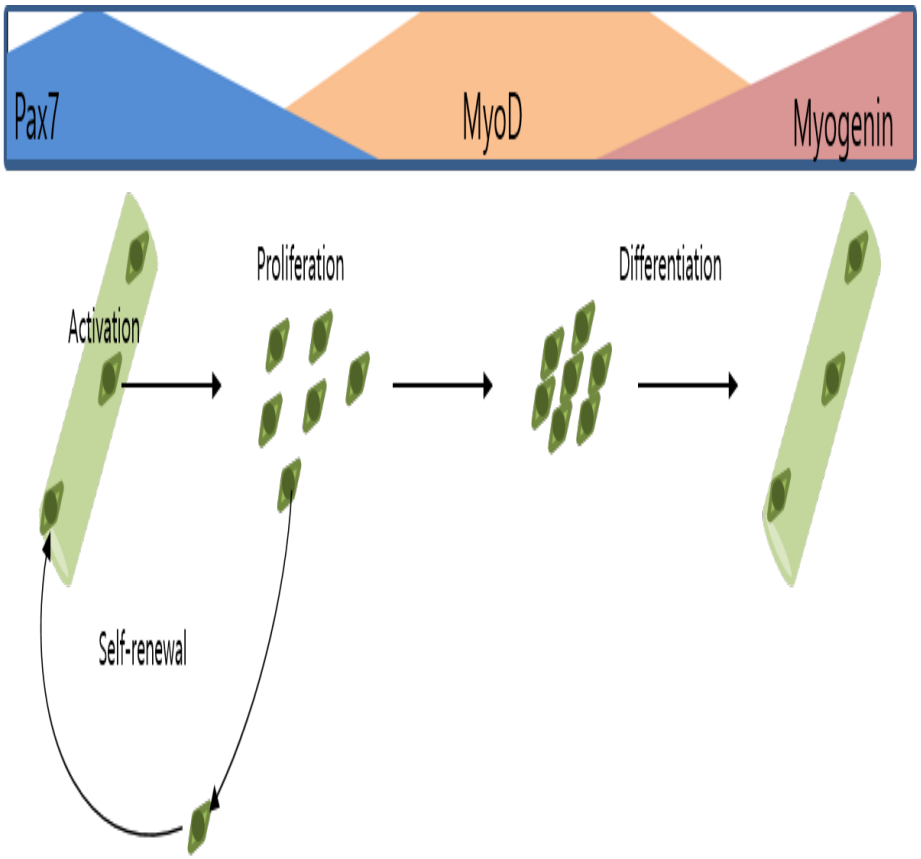

Figure 1 Gene expression patterns of cell proliferation and differentiation markers during myoblast differentiation

To form muscles, satellite cells should be activated and proliferated, differentiated. In quiescent satellite cell stage, Pax7 protein is expressed at high levels. When satellite cells are activated by stimuli, proliferation begins. After activated, the expression of Pax7 decreases and the expression of MyoD increases. Then, the satellite cells are differentiated, up-regulate Myogenin and down-regulate Pax7. In addition, a small cell population in the proliferation stage does not up-regulate Myogenin. This population becomes the self-renewal stage via up-regulation of Pax7 and down-regulation of MyoD.

3. Knockdown using miRNA

1) Knockdown

Knockdown is a procedure that the expression of specific genes is reduced. This procedure is proceeded by genetic modification and treatment with a short DNA or RNA oligonucleotide which has a sequence complementary to the target gene or mRNA transcript (Summerton, 2007).

Transient knockdown and RNAi are typical to knockdown system. Transient knockdown is one of the results of genetic modification. It is occurred by an oligonucleotide binding to an mRNA or a template gene. This causes a temporary change in gene expression without any modification of the chromosomal DNA (Summerton, 2007, Summerton, 1999). In a transient knockdown, the binding of

oligonucleotide leads to decreases of expression by various processes such as the blocking of transcription, translation, the degradation of mRNA transcript and using nuclease cleavage sites for maturation of functional RNA, including miRNA (Nasevicius and Ekker, 2000, Finckbeiner et al., 2011).

Degradation of mRNA is one way of the gene silences and known as RNAi. RNA interference (RNAi) is a procedure that RNA inhibits in gene expression or translation by targeting mRNA molecules. RNAi used to name in various terms; post-transcriptional gene silencing (PTGS), co-suppression. Andrew Fire and Craig C. Mello's work contribute to comprehensive understandings of RNAi in the nematode worm, *Caenorhabditis elegans* (Fire et al., 1998). They received the 2006 Novel Prize in Physiology or Medicine for this achievement. Since RNAi was fully studied, it has been used for the gene

suppression precisely, efficiently and stably (Saurabh et al., 2014). There are two types of small RNA molecules; microRNA (miRNA), small interfering RNA (siRNA). These can bind specific messenger RNA (mRNA) and regulate their activity. In organism, RNAi has an important role for defending nucleotide sequences that are viruses and transposons.

RNAi is regulated by a RNA-induced silencing complex (RISC) and is initiated by Dicer. Dicer is an enzyme that cleaves long double-stranded RNA (dsRNA) molecules into short double-stranded of siRNA. Short double-stranded RNA interact with the argonaute protein which is one of components of RISC (Macrae et al., 2006, Oliver et al., 2014). When an organism is infected with an engineered virus or other systems (Pfeffer et al., 2004). in vitro (exogenous), the RNA is directly degraded by the Dicer into a short double-

stranded RNA in the cytoplasm. Then, the dsRNA can also be endogenous, like pre-microRNA expressed from RNA-coding genes in living cell. Transcripts of these genes are formed in the stem-loop structure, which is a characteristic of pre-miRNA in nucleus. Then they are exported to the cytoplasm (Bagasra and Prilliman, 2004).

miRNAs are encoded in non-coding RNAs, which help regulation of gene expression specially during development (Wang and Li, 2007, Zhao and Srivastava, 2007, Miyaki and Asahara, 2012). Matured miRNAs are similar to siRNAs are produced from exogenous dsRNA using methods such as transposon system. However, miRNAs must undergo extensive post-transcriptional modification to matured one (Gregory et al., 2006). In the cell nucleus, pre-miRNA encoded in RNA-coding gene is processed and linked to stem-loop structure by a microprocessor complex (Qureshi et al., 2014, Pillai et al., 2007) .

This dsRNA of pre-miRNA is maturated by Dicer and is integrated into the RISC complex. Eventually, siRNA and miRNA use the same downstream cellular mechanism (Okamura et al., 2004, Lee et al., 2004).

2) Knockdown using miRNA

In this study, The *FOXO3* gene was modified by the miRNA to identified pCM Foxo3 knockdown cell lines. miRNA is a small non-coding RNA molecule that is suitable for the usage of a knockdown system because RNAi can function post-transcriptional regulation and RNA silencing (Ambros, 2004, Bartel, 2004).

There are three processes that are mRNA molecules silenced by specific binding of miRNA; reducing translation efficient of the mRNA into protein, cleaving of the mRNA strand, shortening of poly(A) tail

of mRNA (Bartel, 2009, Fabian et al., 2010). There is a difference that miRNAs are derived from regions of the RNA transcript which folds themselves to form short hairpins, while siRNAs are derived from longer regions of dsRNA (Friedman et al., 2009, Lagos-Quintana et al., 2002).

Endogenously expressed miRNAs control translational repression and regulation of development, especially maintenance undifferentiation stage and the time point of development in incompletely differentiated cell (Saumet and Lecellier, 2006, Carrington and Ambros, 2003, Palatnik et al., 2003). Therefore, miRNA can be used as a knockdown procedure by modifying the expression of key regulation genes such as transcription factors, which regulates entire gene networks during development.

Chapter 3. Materials and Methods

1. *FOXO3* knockdown using miRNA

1) Construction of vectors

To knockdown of the chicken *Forkhead Box O3 (FOXO3)* gene, two miRNAs was inserted into the *piggyBac* transposon transgene expression system vector (System Biosciences, Palo Alto, CA, USA) after *Asc* I double digestion and ligation. (*piggyBac* CMV-GFP-miRNAs) First, the transgene expression vector carrying the CMV-eGFP gene was inserted into the 5' - and 3' -UTR *piggyBac* transposon elements and miRNAs were regulated by the cytomegalovirus (CMV) promoter. The promoter of elongation factor

1 (EF1) was used for expression of the puromycin-resistance gene in the transgene expression vector. The miRNAs inserted into the vector are synthesized by combining chicken Foxo3 miRNA-1 (5'-gct ggt gac ggt ctc cat gga caa gag caa cac gct gtg aca ctt caa act tgt tgc tgt tgt cca tgg aca ctg tgg tca gca-3') and chicken Foxo3 miRNA-2 (5'-gct ggt gac gca cac tat tca gga caa caa gcc gct gtg aca ctt caa act gct tgt tct cct gaa tag tct gtg tgg tca gca-3') (Bionics, Seongdong-gu, Seoul, Republic of Korea).

2. Cell culture

1) pCM cell culture.

pCM cells were maintained and passaged in Medium 199 (Invitrogen), supplemented with 10 % fetal bovine serum (FBS;

HyClone), 2 % chicken serum (Sigma-Aldrich) and 1 × antibiotic-antimycotic (Invitrogen) and subcultured at 70 % confluency. These cells were cultured in an incubator 37 ℃ in an atmosphere of 5 % CO_2 and 60-70 % relative humidity.

2) Transfection and Selection

To establish the transgene expressing cell lines, transgene expression vector was co-transfected with *piggyBac* transposase using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. Once the seeded cells reached 80 % confluency on 6-well culture plates, they were washed with phosphate-buffered saline (PBS) and refreshed with 2 ㎖ of the culture media without antibiotic-antimycotic. The plasmid DNA-lipid complex consisting of 7.5 ㎕ Lipofectamine 3000 reagent in 250 ㎕

Opti-MEM (Invitrogen) and 10 ㎕ P3000 reagent with 2.5 ㎍ *piggyBac* transgene vector and *piggyBac* transposase plasmid in 250 ㎕ Opti-MEM was added to each well. One day after lipofection, 10 ㎍ puromycin $㎖^{-1}$ was added to select the cells stably transfected with the transgene.

3) Induction of myotube differentiation

To induce myotube differentiation at 80 % confluency of cells, after washed one time by using PBS, the differentiation medium containing 0.5 % FBS and 1 × antibiotic-antimycotic was changed. The differentiation medium was replaced with fresh differentiation medium daily.

3. Functional assay

1) Quantitative RT-PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was quantified using a NanoDrop 2000 (Thermo Scientific), and 2 ㎍ RNA were used for cDNA synthesis using random primers (Invitrogen) under standard conditions. Each 20 ㎕ RT-PCR reaction mix contained 2 ㎕ cDNA, 2.5 ㎕ PCR buffer, 1 ㎕ dNTP mixture (2.5 mM), 1unit Taq DNA polymerase, and 10pmol forward and reverse primer (Table 1). Quantitative RT-PCR analysis was performed using the iCycler iQ Real-time PCR detection system (Bio-Rad) and EvaGreen (Biotium, Fremont, CA, USA). The PCR parameters were as follows: an initial incubation at 94 ℃ for 5 min,

followed by 40 cycles at each condition (Table 1). The reaction was terminated by a final incubation at 72 ℃ for 10 min, and melting curve profiles were analyzed for the amplicons.

2) Western blotting

Total protein was extracted with 1 × radioimmunoprecipitation (RIPA) lysis buffer and separated on a 10 % polyacrylamide gel followed by transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The primary antibodies used were mouse anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA), anti-Foxo3 (Invitrogen), anti-Pax7 (R&D Systems, Minneapoils, MN, USA), anti-MyoD (Santa Cruz Biotechnology), anti-Desmin (Novus Biologicals, Littleton, CO). HRP-conjugated anti-mouse IgG or anti-rabbit IgG (Bio-Rad) were used as secondary antibodies. The blots

were treated with ECL substrate solutions and exposed in a ChemiDoc XRS System (Bio-Rad) to detect chemiluminescence.

3) Cell growth curve

To perform cell growth curve, cell was subcultured in 24-well culture plates (2×10^4 cell / well). Count the cell number at the same time from the next day to the fifth day. When counting cell, count the number of cells in the 4-well culture plates and average them. Make cell growth curve using the data for 5 days.

4) Statistical analysis

Statistical analysis was performed using the Student's t-test in SAS version 9.3 software (SAS Institute, Cary, NC, USA). Significant differences between the groups were determined using the general

linear model (PROC-GLM) in SAS software, Differences between treatments were deemed significant when the P value was less than 0.05.

5) Cell imaging

To take cell images, cells were subcultured in 12-well culture plates (4×10^4 cell / well). Using Cytation^{TM} imaging Readers (BioTek, Winooski, VT, USA), take images of regular pCM and pCM Foxo3 knockdown cells for 5 days. Images were taken every 20 min for 5 days.

Chapter 4. Results

1. miRNA-mediated *FOXO3* knockdown in chicken myoblast cells

1) Construction of vectors for *FOXO3* knockdown

We found *FOXO3* gene and chicken genome sequence information [NCBI (http://www.ncbi.nlm.nih.gov), *Gallus gallus FOXO3*, XM_001234495 and UCSC Genomics Institute, chicken BLAST (http://genome.ucsc.edu)]. We designed and constructed *piggybac* transposon vector that can express the GFP-miRNAs controlled by the CMV promoter (Figure 2A). miRNAs had target and mature form sequences (Figure 2B). miRNAs were synthesized and two sequences of miRNAs are maturated and formed hairpin structures

(Figure 2C). After transfection, *piggyBac* CMV-GFP-miRNAs expression vector was expressed in pCM cells and cells with puromycin resistance were identified by treatment of puromycin reagents. Sorted Foxo3 KD cells are used in the following experiments.

(A)

piggyBac CMV-GFP-miRNAs

(B)

```
-. Chicken Foxo3-1 target       : GTGTCCATGGACAACAGCAACAA
   Chicken Foxo3-1 mature form : ttgttgctgttgtccatggacac
-. Chicken Foxo3-2 target       : CAGACTATTCAGGAGAACAAGCA
   Chicken Foxo3-2 mature form : tgcttgttctcctgaatagtctg

Foxo3-1:
gctggtgacggtctccatggacaagagcaacacgctgtgacacttcaaacttgttgctgttgtc
catggacactgtggtcagca
Foxo3-2:
gctggtgacgcacactattcaggacaacaagccgctgtgacacttcaaactgcttgttctcctg
aatagtctgtgtggtcagca
```

(C)

```
AscI
ggcgcgccgctggtgacggtctccatggacaagagcaacacgctgtgacacttcaaacttgttg
ctgttgtccatggacactgtggtcagcaCAGGGTGGCTAGAGAAGGACTGGCAAGGGCTAGGAG
ATGGAACAAAACCTGCCTTGTGCACAAGCCCAGGCCCTGCAGGGGTGATAAAGCCTGGCTGTGG
TGTGTGGTGGTCAGGTCTGTGGCTGTGCTCTCCGTCATCATCGTCATTCTTCCTGTGGGGCACA
TCCATCCGGAGCCACAAGGAGCATCAGGAGCTgctggtgacgcacactattcaggacaacaagc
cgctgtgacacttcaaactgcttgttctcctgaatagtctgtgtggtcagcaCTGGCATCACGT
GGGCAACGCCTGGGAGAGCCATCTACATGCGTCCCAAACACTGCCTGCTTTTGCCCTGTGTCTG
CAGCAGGGCTCACCATGGGACAGGGAAGACATGAGTGCTAACTCATCTGTTGACAGCAAAGGCA
CGAAAGGATATTTTGTCCTTGAAGCATCACCCCACAGCCTAAGAAACTCCCTCACTTTATTCGG
CGCGCC
                Asc I
```

Figure 2 *FOXO3* gene knockdown in pCM cells.

(A) The Expression vector of *piggyBac* CMV-GFP-miRNAs. The CMV promoter regulated expression of GFP-miRNAs and EF1 promoter regulated expression of puromycin resistance. Termination signal followed after the both promoters. (B) The sequences of two miRNAs. Blue sequences are target sequences of miRNAs and red sequences are mature form sequences of miRNAs. Blue and red sequences form hairpin structures. (C) The sequences and structures of miRNAs. Two miRNAs were needed to synthesize for insert into the *piggyBac* vector.

2. Characterization of pCM Foxo3 KD

1) Characterization of pCM Foxo3 KD in undifferentiated stage.

We used western blotting and qRT-PCR to characterize pCM Foxo3 KD cell lines and to identify effect of pCM Foxo3 KD cells on other genes in the undifferentiated state.

In the undifferentiated stage, the expression of Foxo3 in regular pCM and pCM Foxo3 KD cells was compared by western blotting. It showed significant down-regulation of Foxo3 (Figure 3A). In addition, the expression of MyoD, one of the MRFs, and the expression of Pax7, a critical marker of undifferentiated myoblasts, was down-regulated (Figure 3B). These data demonstrated that the knockdown of Foxo3 worked properly in translational stage and knockdown of Foxo3 influenced expression of MyoD and Pax7.

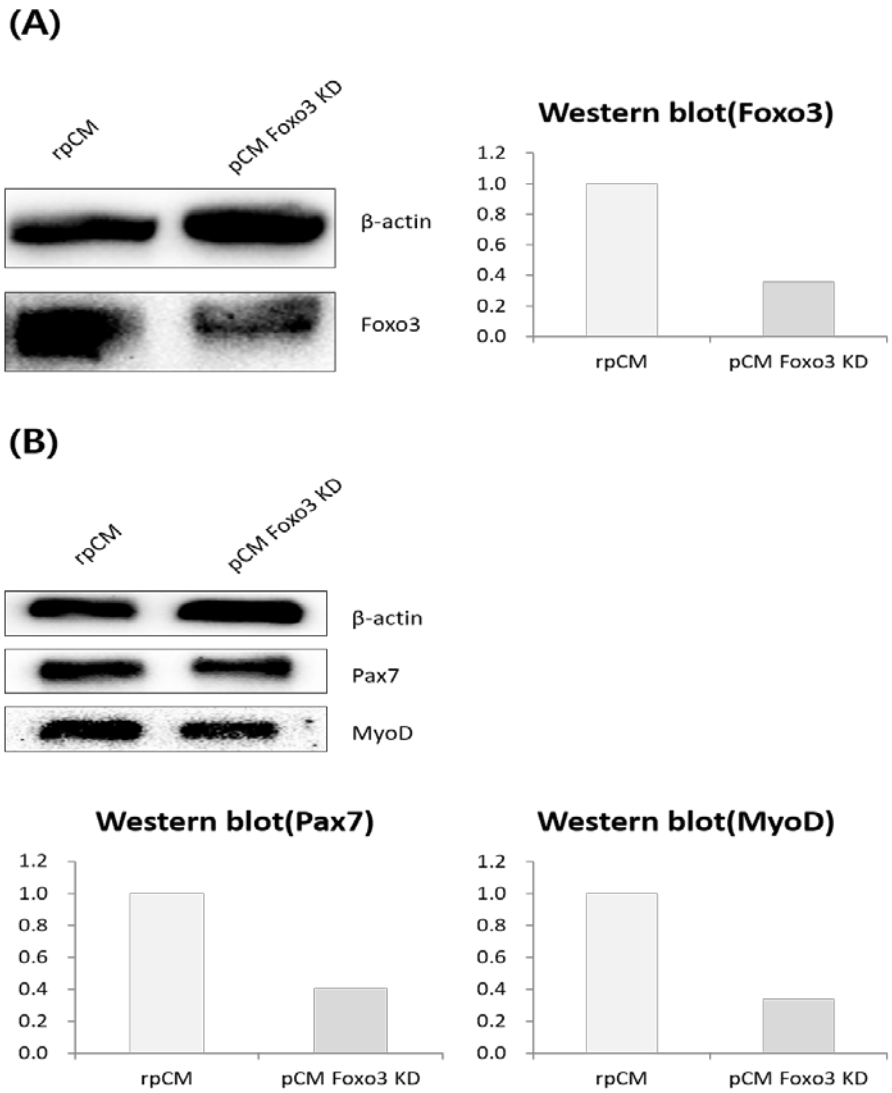

Figure 3 Western blotting of undifferentiated regular pCM and pCM Foxo3 KD cells

(A) The Expression of Foxo3 was down-regulated in the undifferentiated stage. Cell lysates were produced by using cells which were harvested during proliferation. The graph showed statistical analysis of western blotting. (B) The Expression of Pax7 and MyoD was down-regulated in the undifferentiated stage. The graph showed statistical analysis of signal density in western blotting.

Furthermore, to check transcriptional stage of pCM Foxo3 KD cell lines in the undifferentiated state, we used qRT-PCR to analyze the expression of Foxo3 in regular pCM and pCM Foxo3 KD cells, it represented dramatic down-regulation of Foxo3 (Figure 4). Moreover, both the expressions of MyoD and Pax7 were down-regulated (Figure 4).

Additional experiments were performed to check CEBPB and SRF. CEBPB and SRF were also down-regulated (Figure 4). These results showed that pCM Foxo3 KD cell lines down-regulated Foxo3, the target molecule, and also down-regulated MyoD, Pax7, CEBPB and SRF.

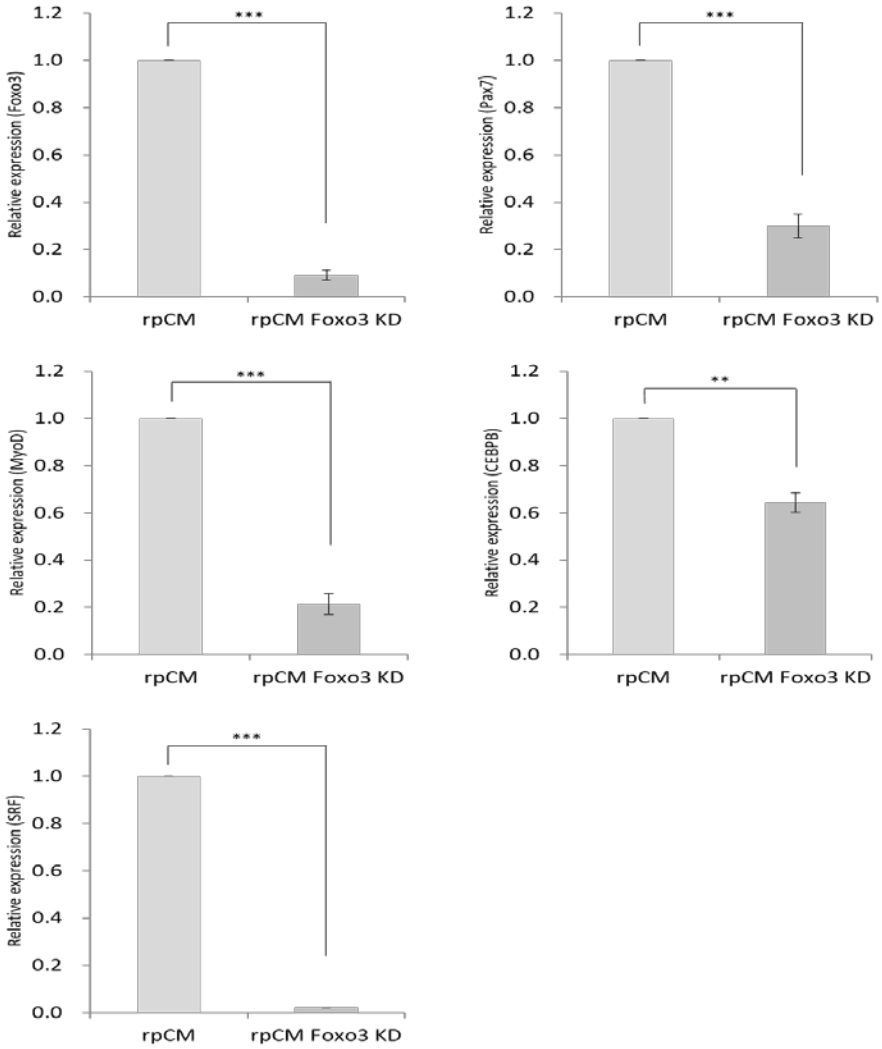

Figure 4 qRT-PCR of undifferentiated regular pCM and pCM Foxo3 KD cells

The Expression of Foxo3, Pax7, MyoD, CEBPB and SRF was compared by qRT-PCR. cDNAs were synthesized by using cells, which were harvested during proliferation. The horizontal axis indicated different cell lines, and the vertical axis indicated relative expression using $2^{-\Delta\Delta Ct}$ value. Every gene was compared with β-actin. Statistical analysis was performed by SAS. *** indicated P < 0.001.

2) Characterization of pCM Foxo3 KD in differentiated stage.

The western blotting and qRT-PCR were performed to investigate characterization of pCM Foxo3 KD cell lines and influence of pCM Foxo3 KD cells on other genes in differentiation state. The regular pCM and pCM Foxo3 KD cells were induced differentiation by low-serum medium. After 4 days of inducing differentiation, the experiments were conducted.

The expression of Foxo3 in regular pCM and pCM Foxo3 KD cells was identified by western blotting. The expression of Foxo3 was significantly down-regulated in differentiated state (Figure 5A). Furthermore, the expression of MyoD, and Pax7 was down-regulated (Figure 5B). However, the Desmin, which is one of the differentiation markers showed the low level of expression in the proliferation stage and high level of expression in differentiation stage

(Figure 5B). Western blot analysis revealed that myogenic terminal protein such as Desmin was expressed high level, although the expression of Foxo3, MyoD and Pax7 was down-regulated in the differentiated state.

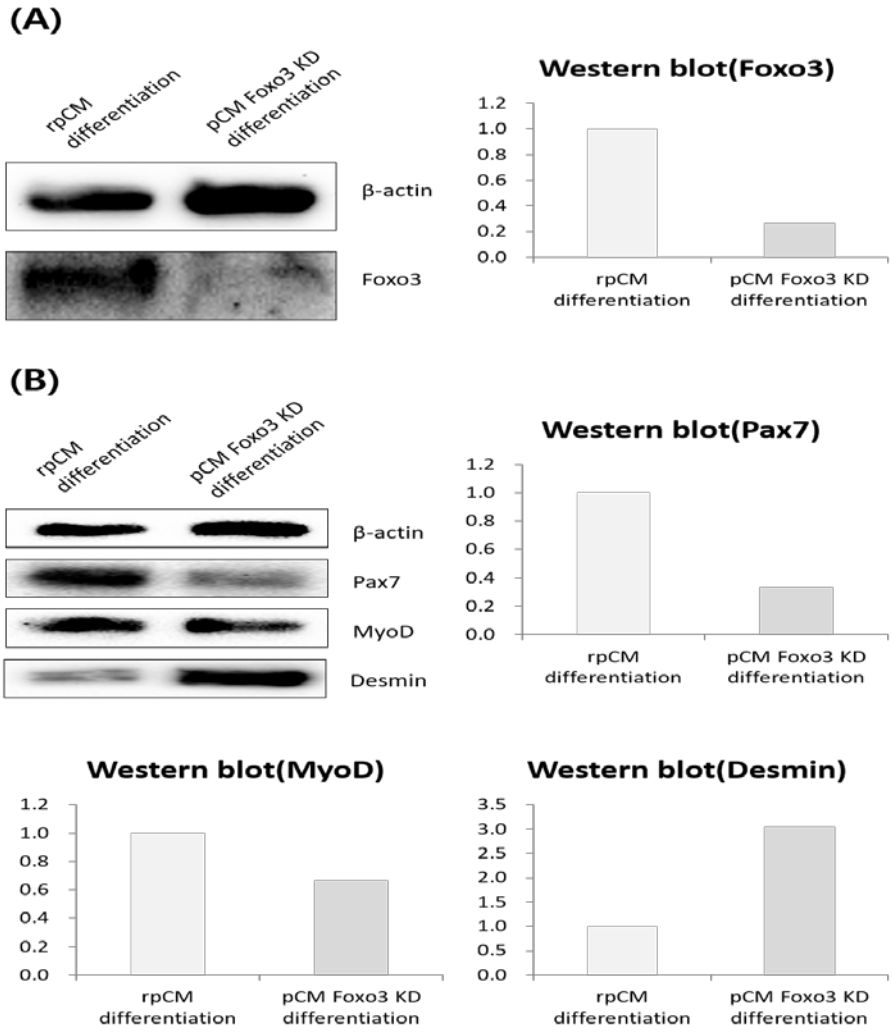

Figure 5 Western blot analysis of differentiated regular pCM and pCM Foxo3 KD cells

(A) The Foxo3 was expressed at low level in the differentiated stage. Cell lysates were produced by using cells, which were harvested after inducing differentiation for 4 days. The statistical analysis of western blotting was showed in the graph. (B) The expression of Pax7, MyoD was down-regulated but, the expression of Desmin was expressed high level in the differentiated stage. The graph showed statistical analysis of signal density in western blotting.

In addition, we performed qRT-PCR to investigate transcriptional expression of regular pCM and pCM Foxo3 KD cell lines in the differentiated state. qRT-PCR data demonstrated significantly down-regulated expression of Foxo3, MyoD, Pax7, CEBPB and SRF (Figure 6). These results were the same as undifferentiated state.

Surprisingly, the expression of Desmin and Myogenin which are myogenic transcripts and terminal differentiation markers was expressed at high level in differentiated stage. We suggested that the expression level of Desmin and Myogenin showed the high level in qRT-PCR analysis and western blot analysis data because the differentiation of pCM Foxo3 KD cells were occurred more than the regular pCM cells.

These results revealed that the pCM Foxo3 KD cell lines in differentiated state down-regulated Foxo3, MyoD, Pax7, CEBPB,

SRF same as undifferentiated state. However, The expression of Desmin and Myogenin was up-regulated in pCM Foxo3 KD cells expectedly.

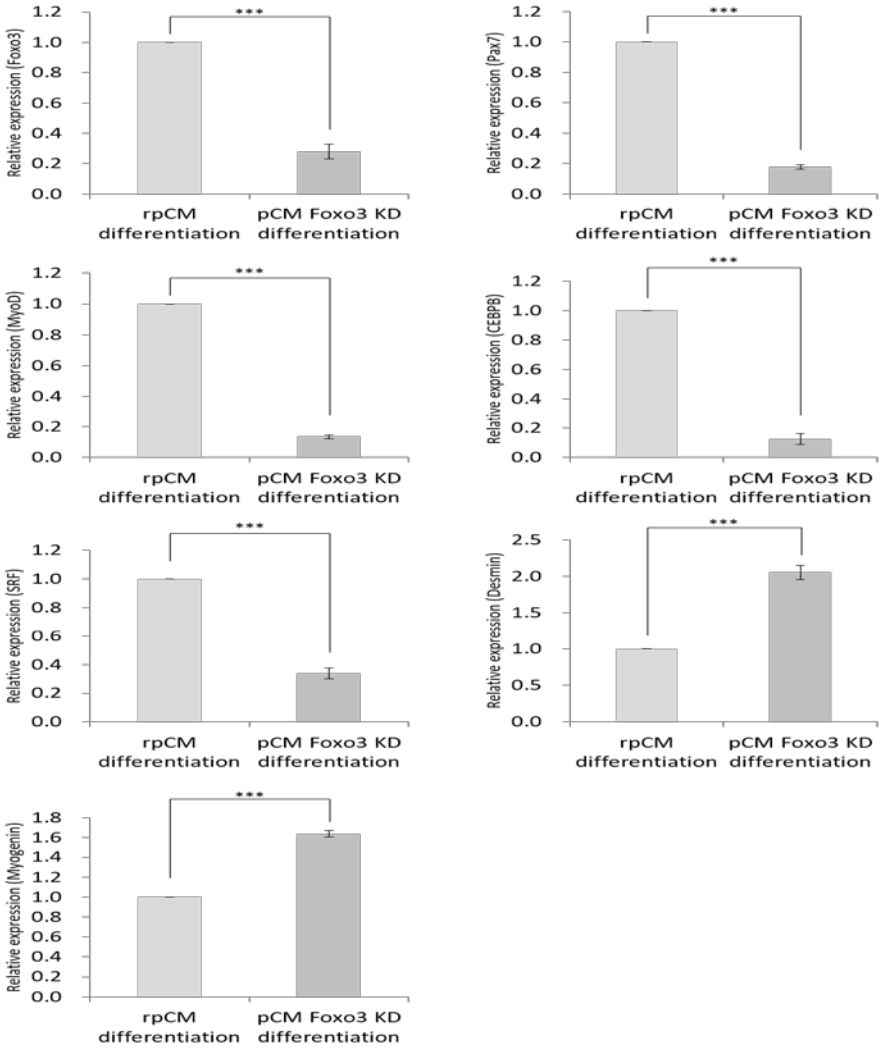

Figure 6 qRT-PCR of differentiated regular pCM and pCM Foxo3 KD cells

The Expression of Foxo3, Pax7, MyoD, CEBPB, SRF, Desmin and Myogenin was analyzed by qRT-PCR. cDNAs were synthesized by using cells, which were harvested after inducing differentiation for 4 days. The horizontal axis indicated different cell lines, and the vertical axis indicated relative expression using $2^{-\Delta\Delta Ct}$ value. Every gene was compared with β-actin. Statistical analysis was estimated by SAS. *** indicated $P < 0.001$.

3. Functional analysis of pCM Foxo3 KD

1) Knockdown of Foxo3 influence growth of chicken myoblasts.

In pCM Foxo3 KD cell lines, the expression of Foxo3 was down-regulated properly, and it influenced other genes which were associated with growth and differentiation of chicken myoblasts. Thus, we decided to identity the functions of knockdown of Foxo3 in growth and differentiation of chicken myoblasts.

To identify influence of the knockdown of Foxo3 in growth of myoblasts, we counted regular pCM and pCM Foxo3 KD cells. Each cells were counted 4-well culture plates at once for 5 days. Cell growth curve revealed that the growth rate of pCM Foxo3 KD cells had the lower level than the regular pCM (Figure 7A). In early stage of growth, day 1 and 2, the growth rates were showed almost the

same total cell number per 1-well. However, the difference of growth rate began to increase from 3 days, and the gap between the growth rate of regular pCM and pCM Foxo3 KD cells became more and more increase on the 4 and 5 days. Because the green fluorescent protein (GFP) was inserted in Foxo3 knockdown vectors to use as one of the selection markers, additional experiment was conducted to identified the influence of GFP in Foxo3 knockdown vectors (Figure 7B). These data indicated that knockdown of Foxo3, not GFP, influenced on growth of chicken myoblasts.

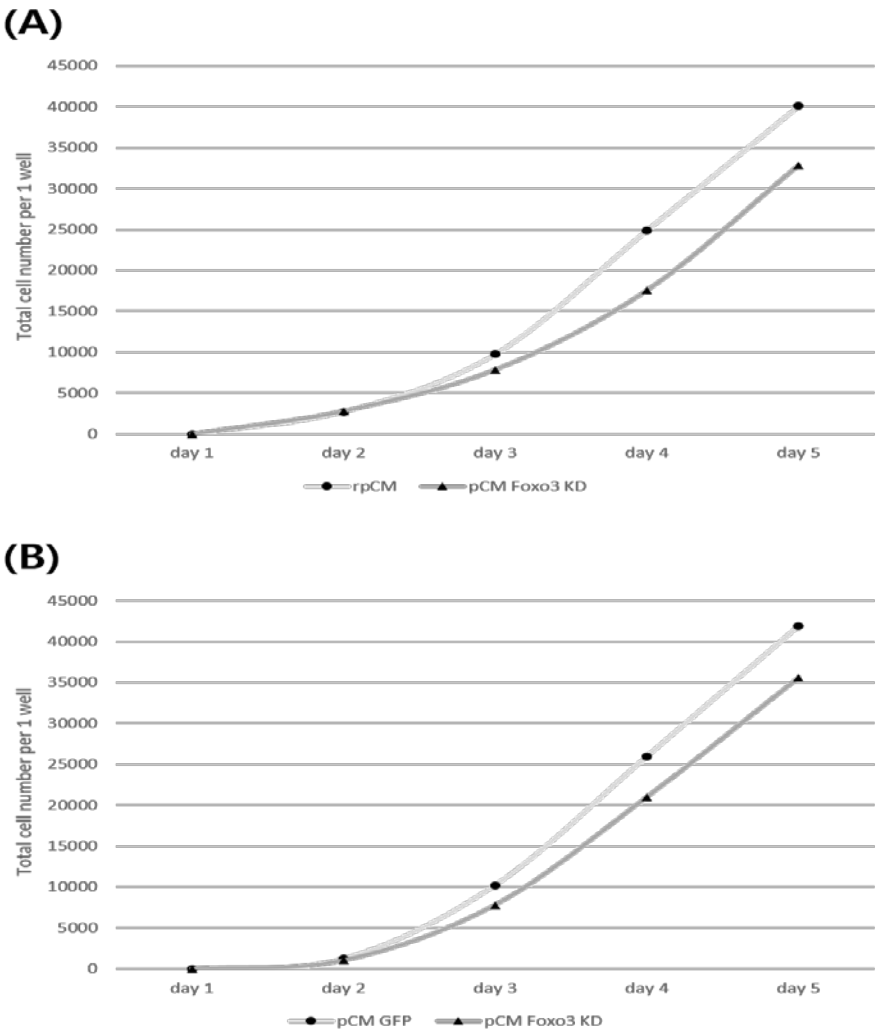

Figure 7 The growth rate of regular pCM and pCM Foxo3 KD cells

The growth rate was analyzed using cell counting data for 5 days. (A) The growth rate between the regular pCM and the pCM Foxo3 KD cells. (B) The growth rates between the pCM GFP and the pCM Foxo3 KD cells. The growth rate of regular pCM cells indicated in black circles (●) and the growth rate of pCM Foxo3 KD cells indicated in black triangles (▲). The horizontal axis indicated the days, the cells were counted and the vertical axis indicated total cell number per 1 well.

2) Knockdown of Foxo3 influence differentiation of chicken myoblasts.

The pCM Foxo3 KD cell lines had the low expression level of Foxo3. Because other genes which were associated with growth and differentiation of chicken myoblasts were affected by down-regulation of Foxo3, functional studies of Foxo3 knockdown were proceeded in differentiation of chicken myoblasts.

Effects of Foxo3 knockdown in differentiation of myoblasts were investigated by using various experiments. First, we took cell images of regular pCM and pCM Foxo3 KD cells for additional experiments (Figure 8). The cell images indicated the morphology of regular pCM and pCM Foxo3 KD cells after inducing differentiation for 4 days. The morphology of pCM cells was fused with several myotubes, but the number of myotubes were lower than pCM Foxo3 KD cells

(Figure 8A). The growth rate of pCM Foxo3 KD cells was lower than regular one, but differentiation of pCM Foxo3 KD cells was occurred more. In pCM Foxo3 KD cells, differentiate myotubes were showed myotubes that were thinner and longer than regular pCM (Figure 8B).

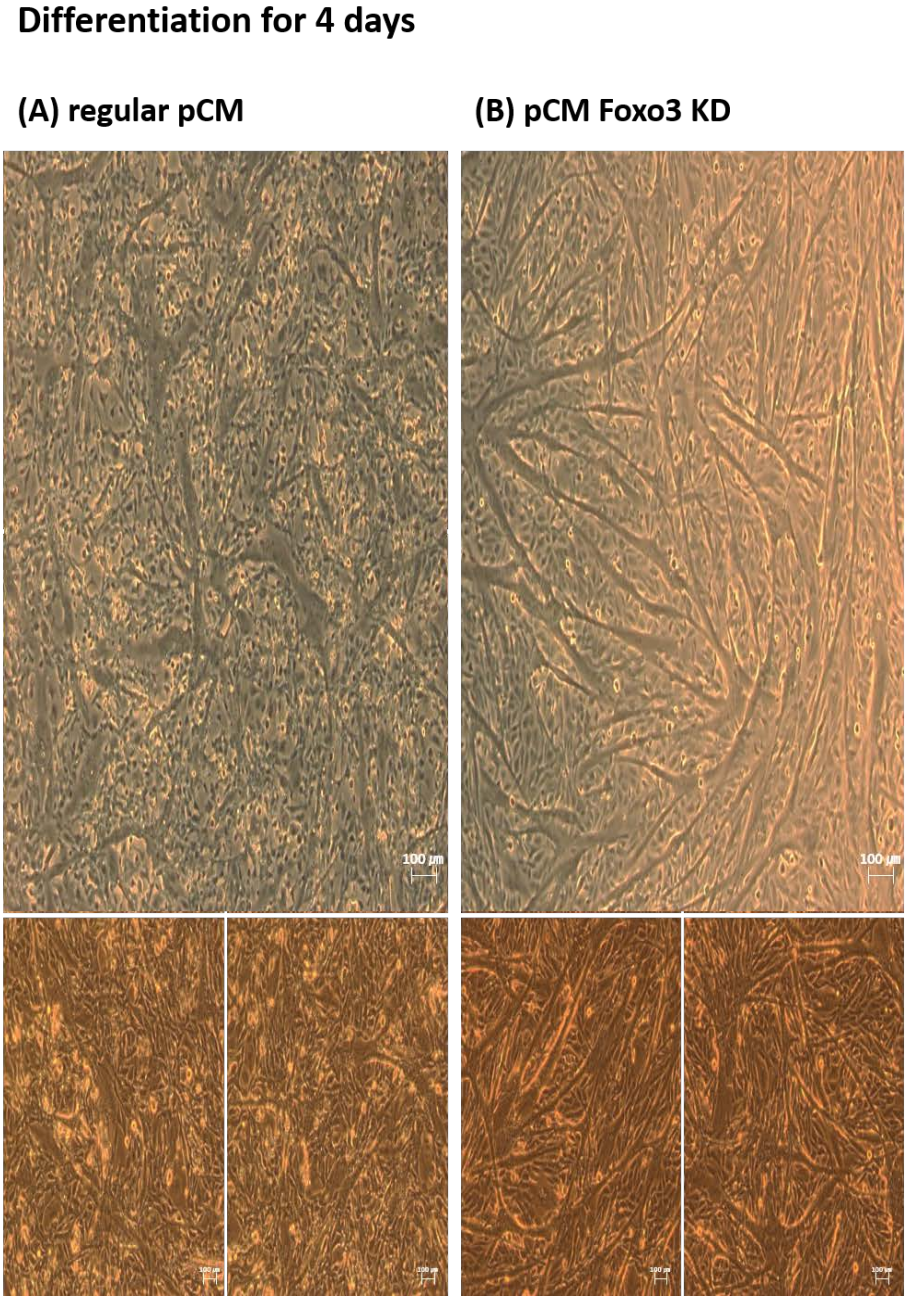

Figure 8 Morphology of regular pCM and pCM Foxo3 KD cells

Images of the cells were taken after inducing differentiation for 4 days. (A) The morphology of regular pCM cells. (B) The morphology of pCM Foxo3 KD cells. The scale bar indicated 100㎛ in white letters.

To observe the number of nuclei and the fusion patterns of cells in differentiated myotubes, regular pCM and pCM Foxo3 KD cells were required to stain in differentiated state by using DAPI staining solution. Thus, regular pCM and pCM Foxo3 KD cells were DAPI-stained after inducing differentiation for 4 days (Figure 9).

The stained cells morphology was demonstrated that cells were fused with other cells in myotubes. In regular pCM, number of fused myotubes were lower than the pCM Foxo3 KD cell lines (Figure 9A), and pCM Foxo3 KD cells had more fused myotubes than the regular one (Figure 9B). However, additional experimentation was needed to determine exact number of myotubes and fused cells.

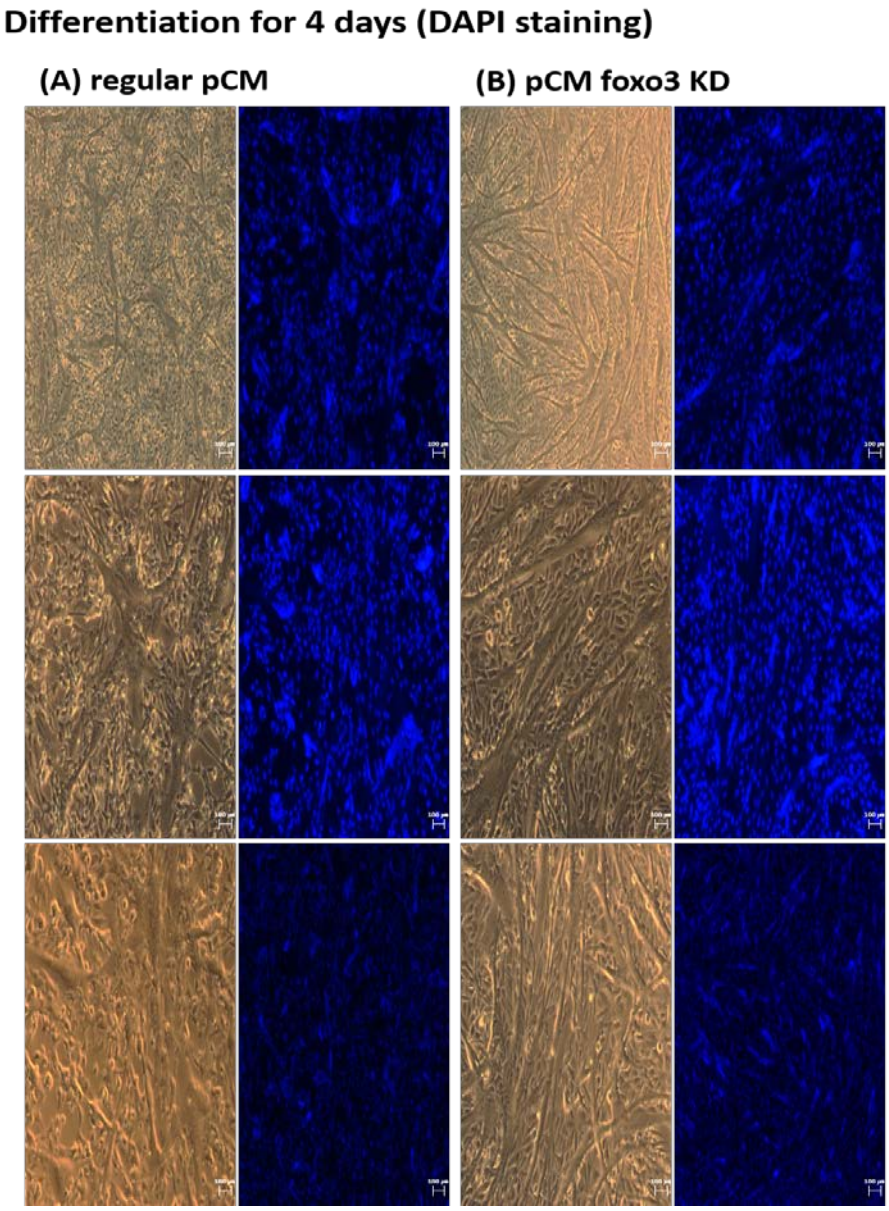

Figure 9 Morphology of DAPI-stained regular pCM and pCM Foxo3 KD cells

To take cell images, the cells were induced to differentiate for 4 days. (A) The DAPI-stained cells morphology of regular pCM cells. (B) The DAPI-stained cells morphology of pCM Foxo3 KD cells. The scale bar indicated 100㎛ in white letters.

After inducing differentiation for 4 days, we calculated percentage of differentiated area, width/length ration, nuclei per differentiated area and initiation time of differentiation to identify exact number of myotubes and fused cells (Figure 10).

To calculate percentage of differentiated area, each cells were counted 14 times in different area in same well of culture plates after inducing differentiation for 4 days. As a result, the pCM Foxo3 KD cells had lager percentage of differentiated area than regular pCM cells (Figure 10A). Uniquely, the shapes of myotubes were different in the pCM and pCM Foxo3 KD cells. Thus, we examined the length and width of myotubes and compared the differences in each other.

The length and width of pCM and pCM Foxo3 KD cells were observed from 20 myotubes in different area of culture plates after inducing differentiation for 4 days. The ration of width/length was

showed that the pCM Foxo3 KD cells had thinner and longer myotubes than regular pCM (Figure 10B).

In addition, we counted nuclei in differentiated area to determine the extent of differentiation in regular pCM and pCM Foxo3 KD cells. Each cells were counted 5 times in different area in culture plate. Unexpectedly, the nuclei per differentiated area were demonstrated that the regular pCM had more nuclei in myotubes than the pCM Foxo3 KD cells (Figure 10C).

As pCM Foxo3 KD cells had more myotubes than regular one and had less nuclei per differentiated area, we decided to examine differentiation starting times. After inducing differentiation, cell imaging system was used to check differentiation starting time in 5-wells of culture plate for 5 days. The initiation of differentiation was revealed that the pCM Foxo3 KD cells were differentiated faster than

the regular one (Figure 10D).

These data suggested that the knockdown of Foxo3 was influenced in differentiation of myoblasts. The pCM Foxo3 KD cells were constructed myotubes earlier and more than regular pCM, but fused myotubes of pCM Foxo3 KD cells had less nuclei in differentiated area and had thinner and longer form than regular pCM.

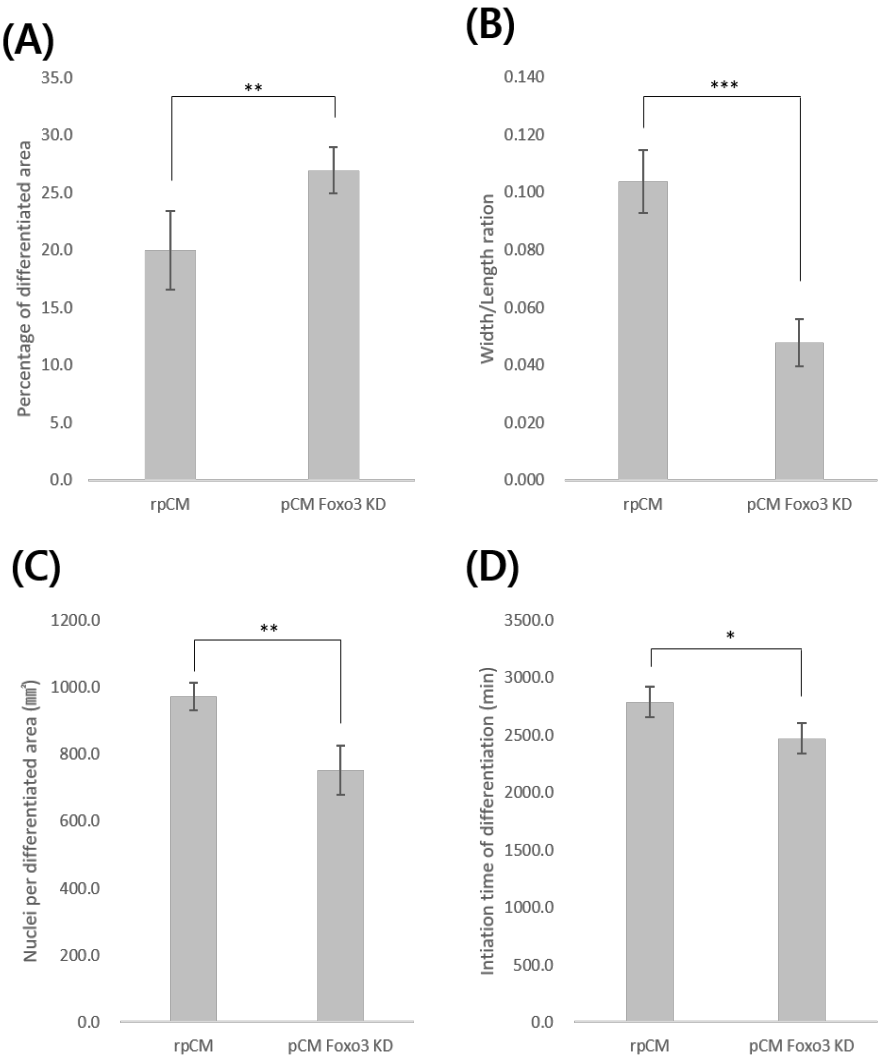

Figure 10 Morphological analysis of regular pCM and pCM Foxo3 KD cells in differentiated stage.

(A) The percentage of differentiated area in regular pCM and pCM Foxo3 KD cells. The horizontal axis indicated types of cells, the vertical axis indicated percentage of differentiated area. The percentages of differentiated area were calculated with the differentiated area/the total area. (B) The width/length ration in regular pCM and pCM Foxo3 KD cells. The types of cells were

indicated in the horizontal axis and width/length ration was indicated in the vertical axis. (C) The nuclei per differentiated area in regular and Foxo3 KD pCM cell lines. The horizontal axis indicated different cells, and the vertical axis indicated nuclei per differentiated area. The nuclei per differentiated area were calculated the number of nuclei in differentiated area/the area of differentiated myotubes. (D) The initiation time of differentiation in regular pCM and pCM Foxo3 KD cells. The horizontal axis indicated types of cells, and the vertical axis indicated initiation of differentiation. The initiation of differentiation was measured in cell images which are taken in every 20 min for 5 days and the initiation of differentiation was determined when it was more than 6 cells fused. Statistical analysis was performed by SAS. * indicated $P < 0.5$, ** indicated $P < 0.01$, *** indicated $P < 0.001$.

Discussion

The *Forkhead Box O3 (FOXO3)* was identified, and proposed to be related to chicken growth (Chen et al., 2015). However, these suggestions were identified in DF1 cells, which were chicken fibroblast cell lines. Thus, there were no data about any influence of Foxo3 to chicken growth in chicken myoblasts. In this study, we investigated functional analysis of Foxo3 by using knockdown procedure during myogenic differentiation in chicken myoblast cells.

In the meantime, we established the first chicken muscle myoblast cell lines. The primary culture cells were isolated form 10 days-old male Hy-Line Brown and became stable cell lines spontaneously. Thus, we decided to investigate the functional analysis of Foxo3

knockdown during myogenic differentiation in chicken myoblast cells established and designed as the first chicken muscle myoblast cell lines, referred to pCM cell lines.

We used miRNA to down-regulate Foxo3 as the knockdown system and *piggyBac* transposon vector as the gene delivery system (Figure 1). The miRNA was one of the smallest RNA molecules and could be used as the RNAi knockdown system (Ambros, 2004, Bartel, 2004, Bartel, 2009, Fabian et al., 2010, Friedman et al., 2009). In this study, miRNA 126-based cassette was used, including two miRNAs for Foxo3 to down-regulate target gene (Chen et al., 2011). The miRNA 126-based cassette had advantages that it could express more than one of miRNAs and exhibit efficient expression level and RNAi activities. The *piggyBac* transposon vector contained efficient and reliable transgene system without any tissue-specific repression

(Park and Han, 2012b, Macdonald et al., 2012).

Our data demonstrated that the knockdown of Foxo3 by using miRNAs work properly thus, the pCM Foxo3 KD cell lines showed down-regulated expression level of Foxo3 in undifferentiated chicken myoblasts. Moreover, knockdown of Foxo3 affected other genes (Figure 3, 4, 11). Besides, the knockdown of Foxo3 showed that the proliferation of chicken myoblasts was reduced (Figure 7). It suggested that the knockdown of Foxo3 has negative effect on growth of chicken myoblasts.

Moreover, the expression of Foxo3 was also down-regulated in differentiated stage of chicken myoblast cells. The knockdown of Foxo3 affected the other genes (Figure 5, 6, 11). The expression of MyoD, Pax7, CEBPB and SRF was down-regulated but the expression of Desmin and Myogenin was up-regulated. Furthermore,

the pCM Foxo3 KD cells had faster initiation time of differentiation, larger percentage of differentiated area, thinner and longer myotubes than the regular pCM cells. However, surprisingly, in the nuclei per differentiated area, regular pCM had more nuclei than the pCM Foxo3 KD cells (Figure 10). Therefore, we suggested that the knockdown of Foxo3 makes growth of cells slowly and forms unusual myotubes when differentiation was induced.

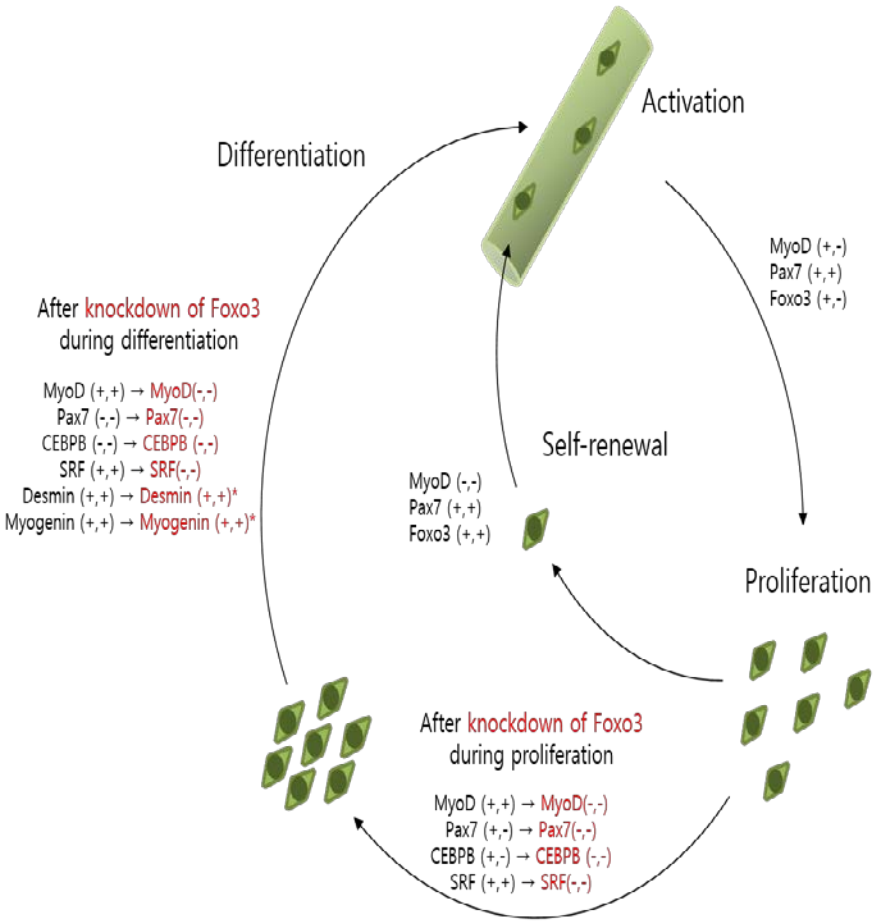

Figure 11 The influence of Foxo3 knockdown in proliferated and differentiated state in chicken myoblast cells

This figure showed the expression of genes during muscle generation. After knockdown of Foxo3, the influence of Foxo3 knockdown was showed in red letter. * indicated over expression than the control.

In summary, we showed the functional analysis of *FOXO3* during myogenic differentiation in chicken myoblast cells by using miRNA knockdown system. The knockdown of Foxo3 made chicken myoblast cells proliferate slowly, differentiate rapidly with induced condition and form myotubes unusual.

We suggested that the growth of pCM Foxo3 KD cells was reduced because the knockdown of Foxo3 brought about a decrease in Pax7 and decrease in MyoD, which is activated by both (Hu et al., 2008). In addition, muscle development by IL-4, IL-6 was decreased as the expression of SRF, which up-regulates muscle growth was reduced due to the knockdown of Foxo3 (Schiaffino et al., 2013). The down-regulated Foxo3 influenced MyoD, Pax7, CEBPB and SRF in undifferentiated and differentiated state indeed.

In addition, in the induced differentiated stage, the effect of Foxo3

made cells differentiate rapidly. We proposed that the pCM Foxo3 KD cell were differentiated faster than the regular pCM because the knockdown of Foxo3 down-regulated target genes of Notch signal such as *Hes1, Hes2, Hes6, HeyL* (Gopinath et al., 2014). In addition, the decrease of CEBPB expression via the knockdown of Foxo3 may cause precocious differentiation (Marchildon et al., 2012). Therefore, the Desmin and Myogenin, terminal differentiation markers, were expressed at high level in transcriptional and translational stage.

Moreover, the down-regulated Foxo3 brought about the unusual myotubes, which were composed of thin and long forms and the low number of nuclei in differentiated area. We suggested that the reasons of unusual myotubes were an increase of atrogin-1, which was related muscle atrophy due to the knockdown of Foxo3 (Chen et al., 2015). The increased atrogin-1 brought about the atrophic

muscle, which showed long and thin shapes of myotubes and low number of nuclei in differentiated area (Sandri et al., 2004).

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Abstract in Korean

가금 산업에서 닭의 성장률은 여러가지 방법으로 경제적인 이점에 관련되어 있기 때문에 중요하게 여겨진다. 또한, 근육의 성장과 분화의 조절인자 역할을 하는 특이적인 유전자와 단백질을 조사하는 것은 선발 육종에 대한 접근법으로 도입되었다. 그래서 우리는 이번 연구에서 닭 근아 세포주에서 근육 분화 간 *FOXO3* 유전자의 기능적인 분석을 진행하였다. 우리는 10일령의 Hy-Line Brown 수컷의 가슴 근육으로부터 pCM 세포주를 확립하였다. pCM 세포주는 안정적이고 무한증식성이 있어 계속적으로 계대배양이 가능한 닭 근아 세포주이다. 두 개의 miRNA와 *piggyBac* transposon vector로 구성된 분자들을 이용하여 *FOXO3* 유전자의 RNA를 방해하여 발현을 하향 조절하였다. 그리고 근육 분화에 따른 Foxo3의 기능과 영향을 분석하기 위해 Foxo3 knockdown 세포주를 동정하였다. pCM Foxo3 knockdown 세포주는

Foxo3의 발현이 극명하게 하향 조절된 것을 보였다. 미분화 상태에서 하향 조절된 Foxo3는 닭 근아세포의 성장과 분화에 연관된 다른 유전자들의 발현을 감소시켰다. 더욱이 분화 상태에서도 Foxo3, MyoD, Pax7, CEBPB 그리고 SRF의 발현은 하향 조절되었다. 그러나, 분화의 지표인 Desmin과 Myogenin의 경우에는 높은 수준으로 발현되었다. 게다가, 하향 조절된 Foxo3는 닭 근아세포의 성장을 느리게 만들고, 분화가 유도되었을 때 분화를 빠르게 만들고, 일반적인 pCM 세포들보다 분화 면적당 핵의 수가 더 적고, 더 얇고 긴 모양의 특이한 근관 세포를 만들었다. 이런 결과들은, Foxo3가 다른 전사 인자들 간의 상호작용을 통해 닭 근아 세포주의 성장과 분화에 연관되어 있다는 것을 보여주었다. 결과적으로 *FOXO3* 유전자는 가금 산업에서 선발 육종의 접근법으로 사용될 수도 있다.

키워드: Foxo3, miRNA, knockdown, 근육 분화, 닭 근아 세포주