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A method for growing and differentiating the C2C12 muscle cell line in the laboratory

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Abstract

Skeletal muscle-related studies have recently been applied in the combat of obesity and metabolic disorders. Culture skeletal muscle cells in in vitro plays an important role in being a promising model for those researches. In the present study, the C2C12 skeletal muscle cells were grown and differentiated in in vitro. The C2C12 myoblasts were grown in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) for 4 – 5 days. The cells then reached confluent about 70% to 100% and the medium was shifted to DMEM plus 2% horse serum which leads the grown C2C12 cells becoming to differentiated myotubes. *Myogenin* mRNA levels were found to be significantly higher in the differentiated myotubes than those in the growing cells confirming the successful formation of the differentiated C2C12 cells. These results indicate that the C2C12 cell line is suitable for culture in in vitro to mimic a skeletal muscle microenvironment for further investigations.

Keywords: C2C12 skeletal muscle cells, culture, differentiation, *Myogenin*

1. Introduction

Obesity and obesity related metabolic disorders such as cardiovascular diseases, fatty liver diseases, and type II diabetes are increasing rapidly and being a health issue of global concern, including Vietnam [1], [2]. Obesity is characterized by hypertrophy of white adipose tissues which are associated with metabolic complications [3], [4]. On another hand, skeletal muscle tissue takes up a large percentage of body mass and, thus, dysfunction of this tissue has been shown to be closely

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associated with obesity and obesity related metabolic disorders [5]. Consistent with this, skeletal muscle dystrophy leads to a higher risk of insulin resistant and type II diabetes in human and mice [6], [7]. Additionally, obesity-related nonalcoholic fatty liver diseases are accompanied with skeletal muscle metabolic dysfunction [8]. These studies prove that skeletal muscle is a key tissue for the regulation of metabolic homeostasis. Skeletal muscle is responsible for the majority of glucose uptake in the body, and it is also a major site of fatty acid oxidation. Thus, improving skeletal muscle metabolism can help prevent and treat obesity and related metabolic diseases such as fatty liver diseases, type 2 diabetes, and cardiovascular disease. A recent study has indicated that exercise can also increase muscle mass which improves skeletal muscle metabolism, as it promotes glucose uptake and fatty acid oxidation, and thus contributes to increased insulin sensitivity [9]. Interestingly, supplements of dietary components also improve skeletal muscle metabolic phenotype and give positive effects on whole system metabolic homeostasis. For example, dietary supplemented with resveratrol, a nature polyphenolic chemical, can directly alter skeletal muscle development and metabolic phenotype which are accompanied by the lowered risks of the high-fat diet induced metabolic disorders in the mice [10]. Taken together, those aforementioned data have suggested that manipulation of skeletal muscle development and metabolism is a strategy to combat against obesity and its related diseases.

However, studies of the effect of factors on skeletal muscle physiology in in vivo are usually faced with many challenges because the body is a complex and unified system with many overlapping, network interactions. Therefore, manipulations of in vitro studies together with in vivo are needed to show that a certain factor is acting on the exact tissue cells. There are several skeletal muscle cell lines that are commonly used in in vitro experiments. These include C2C12, L6, HSkMC, and Sol8 skeletal muscle cell lines [11], [12]. Among them, the C2C12 is a mouse skeletal muscle cell line that is one of the most commonly used cell lines for skeletal muscle studies because it has several advantages over other cell lines, such as (1) Easy to culture: C2C12 cells are relatively easy to culture and maintain in the laboratory, and they can be grown in a variety of culture media; (2) Well-characterized: C2C12 cells have been extensively characterized, and there is a wealth of information available on their behaviour and properties. This makes it easier for researchers to design experiments and interpret results; (3) Availability: C2C12 cells are widely available from commercial suppliers and can be easily obtained for use in experiments; (4) Genetic manipulation: C2C12 cells can be genetically manipulated using a variety of techniques, such as transfection or CRISPR/Cas9 gene editing, to study the function of specific genes or signalling pathways in muscle development and function [13], [14]. Overall, the C2C12 cell line is a valuable tool for studying skeletal muscle development, function, and metabolism in vitro, and its ease of use and well-characterized properties make it a popular choice among researchers. As a result, the present study was performed to conduct a viable method to culture C2C12 skeletal muscle cells from the growth stage to the differentiated adult stage. This provides an in vitro skeletal muscle cell culture model for further studies, especially modelling the effects of obesity and metabolic disorders in in vitro.

2. Experimental Methods

2.1. Cell Culture

The mouse myoblast cell line C2C12 was purchased from the American Type Culture Collection (ATCC CRL-1772, USA). Each vial containing 3 mL freezing soluble of C2C12 myoblasts (10^7

cells/mL) was stored at -20°C in the refrigerator. Before culture, a vial was taken out the freezer and thawed at room temperature from 30 minute to 1 hour. Then, the cells were diluted in the growing medium (5×10^5 cells/mL) and culture in the incubator at 37°C in 5% CO_2. Every day, the culture cells were checked using the light microscopic and the culture medium was newly changed. When the growing C2C12 cells reached about 70% - 100% confluent the growing medium was switched to the differentiated medium [15]. The pictures were taken at time points of the experiment using the microscopic camera (Olympus, Japan). The experiments were conducted in the Department of Food science and Nutrition, University of Ulsan, South Korea.

2.2. Quantitative Real Time PCR (qRT-PCR)

At each time point, the experimental cells were collected for PCR. The cells were washed twice to three times with PBS and lysed in Trizol reagent (Invitrogen, USA) and the final mixtures were collected into 1.5 mL eppendorfs. Two microgram aliquots of total RNA extracted from the lysed C2C12 cells were reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega, USA). Then, the cDNAs were carried out in duplicate with a SYBR premix Ex Taq kit (Takara Bio Inc., USA) by using a Thermal Cycler Dice (Takara Bio Inc., Japan). All reactions were carried out with the same condition: 95°C for 10 second (s) followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Results were read with Real Time System TP800 software (Takara Bio Inc., Japan) and all values were normalized to the levels of the control gene *β-actin*. Thus, for each sample the mRNA expression level of its *Myogenin* was evaluated by divided by its *β-actin* mRNA level. The primers used in the Real Time PCR are listed in Table 1 [16], [17].

Table 1. Mouse primers used for Real Time-PCR.

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Myogenin	TGCCCAGTGAATGCAACTCC	TTGGGCATGGTTTCGTCTGG
β-actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA

2.3. Statistical Analysis

The results are shown as means ± standard error of the mean (SEM). Variables were compared using Student's t-test (Microsoft Excel Software). Comparisons were considered significant difference at $p < 0.05$.

3. Results and Discussion

3.1. Thawing and growing of C2C12 myoblasts

A vial containing 3 mL freezing soluble of C2C12 myoblasts (10^7 cells/mL) was taken out of the -20°C freezer and put outside at room temperature from 30 minute to 1 hour for thawing. After that, the thawed C2C12 cells containing soluble was diluted (5×10^5 cells/mL) in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (the growing medium). This cell containing medium was divided into 4 culture dishes (about 15-20 mL soluble in each dish). Then, those dishes were put in the incubator at 37°C in 5% CO_2. After 1 day, the cells attached to the bottom of the culture dishes and reached about 10% confluence, Figure 1a. The medium was removed, the

cells were washed with phosphate buffered saline (PBS) two times and then the fresh growing medium was added to the dishes. After 2-3 days of incubation, the growing cells reached about 50% confluence, Figure 1b. From this time point, the media in incubated dishes were sucked and washed twice with PBS, then, each 10 mL of PBS containing 0.05% Trysin and 0.02% EDTA was added in the dishes for 2-3 minutes at room temperature. When the time finished, the solution was suctioned, and the dishes were tapped slightly around the walls to completely detach the cells from the bottom of the dishes. Next, 10-15 mL of the fresh growing medium was added to the dishes and pipetting and suctioned, to completely transfer the cells from each two old dishes to a new cultured dish. These new dishes were continuously incubated in the incubator and the cell grew fast to reach about 75% - 100% confluence at day 4-5 (from starting day), Figure 1c. The method to detach the cells and transfer to new cultured dish has been manipulated in culture of several cell lines [18]. This strategy to make the cultured cells growing wells and not losing their differentiation potential [19].

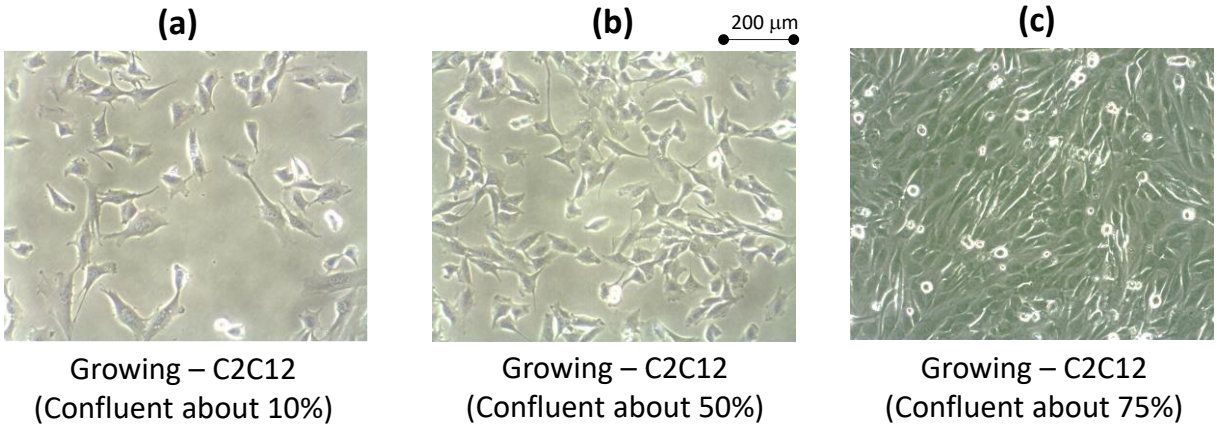

Figure 1. Proliferation process of C2C12 myoblasts. Light microscopy-based images of proliferating C2C12 myoblasts at several time points. (a) The myoblasts grew at day 1. (b) C2C12 myoblasts grew ats day 2-3. (c) C2C12 myoblasts grew at day 4-5. Magnification 100; Scale bar is 200 µm.

3.2. Differentiation of C2C12 myoblasts

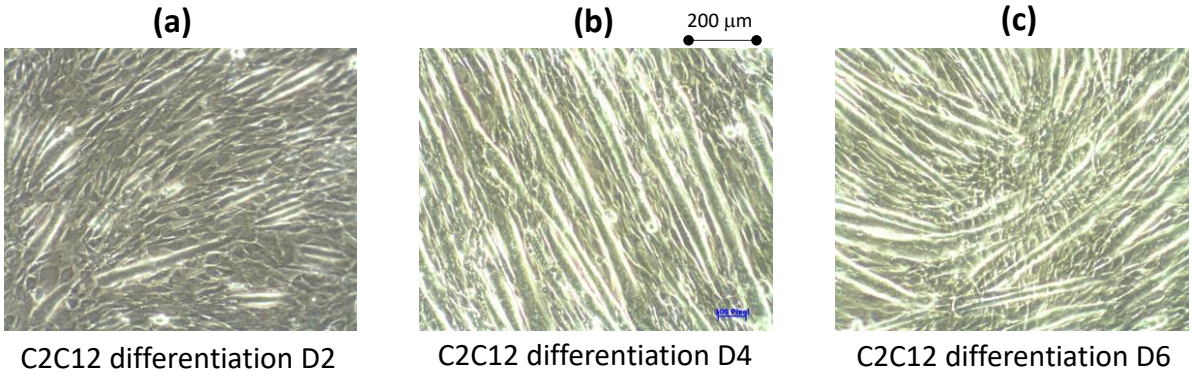

Figure 2. Differentiation process of C2C12 myotubes. Light microscopy-based images of differentiated C2C12 myotubes at several time points. (a) C2C12 myoblasts were differentiated at day 2 (D2). (b) C2C12 myoblasts were differentiated at day 4 (D4). (c) C2C12 myoblasts were differentiated at day 6 (D6). Magnification 100; Scale bar is 200 µm.

Myoblast differentiation is an important period in the development and maintenance of skeletal muscle tissue. In this process, the undifferentiated myoblasts are transformed into mature and functional muscle fibers named myotubes [20]. The confluence of culture cells refers to the percentage of the culture dish covered by adherent culture cells, which can affect the cells' differentiation when switched to a differentiated medium. How much confluence of the culture precursor cells should be used for differentiation remains variable, which depends on several factors such as the cell line, the differentiated medium, and the expected experimental results products [21], [22]. In the present study, when the growing myoblasts reached about 75% confluence the growing medium was replaced by the differentiated medium that consisted of DMEM added with 2% horse serum. The culture dishes were put in the incubator at 37°C in 5% CO_2 and the differentiated medium was changed every 2 days. On day 2 of differentiation, several myoblasts fused to become bigger size myotubes as shown in Figure 2a. On day 4 of differentiation, differentiated myotubes with a typical long cylindrical morphology were almost formed, Figure 2b. On day 6 of differentiation, the differentiated process was likely reached to a peak when morphology of myotubes was markedly transformed and the number of myotubes was likely at maximum, Figure 2c.

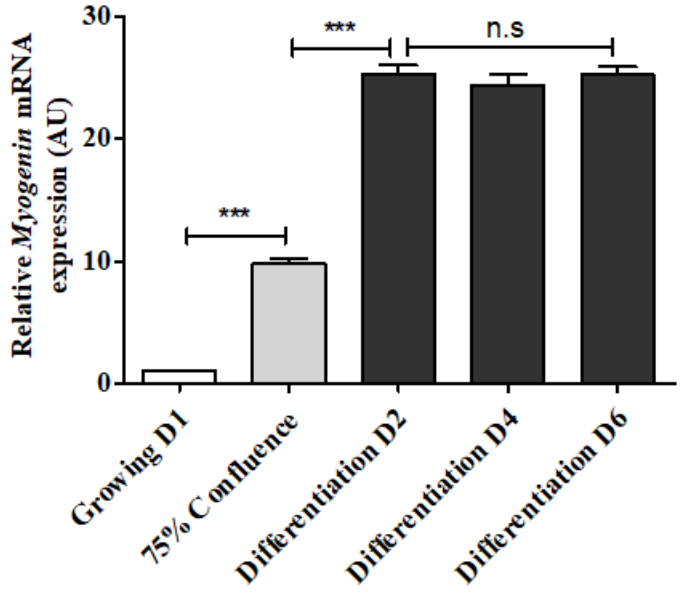

Figure 3. Expression of skeletal muscle marker**.** Expression of *Myogenin* mRNA in C2C12 muscle cells was determined by qRT-PCR at various time points. Quantitative levels of Myogenin mRNA were normalized to the levels of *β-actin*. Data are means ± SEM of three independent triplicate experiments. *** $p < 0.001$ compared between the two groups. n.s is not a significant comparison. AU is an arbitrary unit. The mRNA expression level of *Myogenin* of Growing D1 was considered to have a value of 1, and the mRNA expression levels of *Myogenin* of the other groups were compared to the value 1 of Growing D1 group.

3.3. Determination of C2C12 growing stages

The differentiation of myoblast into mature myotubes is regulated by complex molecular signals and factors. Among them, the myogenic regulatory factors (MRFs) such as Myogenin, MRF4, and MyoD play a crucial role in regulation of myoblast differentiation [23]. Interestingly, a recent study has indicated that Myogenin molecule being as a marker of myogenesis and maturation of muscle cells

[24]. Consistent with those findings, the result of the current study showed that the expression levels of *Myogenin* mRNA in 75% confluent myoblasts were significantly higher than that of growing myoblasts at day 1, Figure 3. Moreover, the expression levels of *Myogenin* mRNA in the cells at differentiation day 2 (D2) were significantly higher than that in myoblasts at 75% confluence, Figure 3. Surprisingly, the expression levels of *Myogenin* mRNA were not significantly different among the differentiated myotubes at day 2, day 4, and day 6, Figure 3. Therefore, the current data suggest that Myogenin molecule is a marker of development periods of skeletal muscle cells at growing, confluent and differentiation stages. The observation of no difference in Myogenin expression among time points of myotube differentiation is also indicated in a recent study [25]. This means that Myogenin is maximum expressed at early time of differentiation, and thus, Myogenin should not be used for determining different time points of mature myotubes.

4. Conclusion

As a result, the data from the present study indicates a method that is capable to do in the laboratory to culture and differentiation of C2C12 skeletal muscle cells becoming typical skeletal muscle fibers which will be further used for potential studies in metabolism and development of skeletal muscle tissue. Additionally, the data of mRNA expression suggest that Myogenin is a marker for distinguishing stages among early growing myoblasts, late growing myoblasts and early differentiate myotubes but not a maker for determining stages in myotube development. Thus, further studies to investigate the marker(s) specific for distinguishing of myotube development stages are needed. A successful model of culture mature skeletal muscle cells would be applied for research in close related fields such as personalize medicine for individual muscle-related disorders, molecular mechanisms underlying various muscle diseases, and the effects of physical exercise on muscle cells.

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