Comparison of Proliferation and Differentiation of Hanwoo Muscle Satellite Cells and C2C12 Myoblast Cells According to Culture Temperature

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Abstract

To improve culture efficiency of Hanwoo muscle satellite cells, these cells were cultured at different temperatures. Hanwoo muscle satellite cells were compared with C2C12 cells to observe proliferation and differentiation at culture temperatures of 37 ℃ and 39 ℃ and determine the possibility of using them as cultured meat. Immunofluorescence staining using Pax7 and Hoechst, both cells cultured at 37 ℃ proliferated better than cultured at 39 ℃ ($p < 0.05$). When differentiated cells were stained with myosin and Hoechst, there was no significant difference in root canal thickness and Fusion index. In Western blotting analysis, Hanwoo muscle satellite cells were no significant difference in the expression of myosin or cytochrome C between cells differentiated at the two temperatures. C2C12 cells were no significant difference in the expression of myosin between cells differentiated at the two temperatrues. In RT-qPCR analysis, Hanwoo muscle satellite cells cultured at 39 ℃ had significantly ($p < 0.05$) higher expression levels of MyHC, MRF4, and myoglobin than those cultured at 37 ℃. C2C12 cells cultured at 39 ℃ showed significantly ($p < 0.05$) higher expression levels of myogenin and myoglobin than those cultured at 37 ℃. To increase culture efficiency of Hanwoo muscle satellite cells, proliferating at 37 ℃ and differentiating at 39 ℃ are appropriate. Since results of temperature differences of Hanwoo muscle satellite cells were similar to those of C2C12 cells, they could be used as a reference for producing cultured meat using Hanwoo satellite cells.

1. Introduction

Prenatal and postnatal skeletal muscle growths in animals are due to embryonic myoblasts and satellite cells, respectively. In particular, muscle satellite cells found between the sarcolemma and the basal lamina participate in muscle development, recovery, and regeneration (Yin et al., 2013). Although they are normally in a resting state, they can be activated by external stimuli such as muscle damage and exercise. Activated satellite cells can repeatedly increase myoblasts through proliferation according to demands of skeletal muscle. Through differentiation, myoblasts can attach to each other to form myotubes and myofibroblasts. By fusion to the skeletal muscle, myoblasts can regenerate and grow (Post, 2012; Lim & Son, 2019). These muscle satellite cells play an important role in muscle regeneration after injury. Among various cell types for cultured meat production, muscle satellite cells have been shown to be the most effective and promising (Yin et al., 2013). Among stem cells, except for satellite cells, there are embryonic stem cells, induced pluripotent stem cells, and adipose-derived adult stem cells. Although these cells have sufficient differentiation capacity (Kadim et al., 2015), whether they can successfully form muscle tissues has not been reported yet.

In 2013, a company made the first cultured beef hamburger. Since then, dozens of companies have entered the cultured meat field, developing a variety of products including chicken, beef, pork, and seafood (Choudhury et al., 2020). In addition, cell-based cultured meat development is being actively carried out in several countries such as the United States, Israel, the Netherlands, Canada, Japan, Singapore, Spain, China, the United Kingdom, and Turkey (Faustman et al, 2020). When cells are cultured under ideal conditions using cell culture technology, they can proliferate up to at least 20 times. A large

amount of muscle fibers can be obtained with only a very small number of cells (Mizuno et al., 2010). Although various cells can be used for the production of cultured meat, C2C12 myoblast is popularly used for cell culture experiments including cultured meat because it rapidly proliferates and differentiates with the advantage of being well cultured in a relatively poor environment (Marloes et al., 2010). Various cattle breeds are being researched for cultured meat production. However, research on cultured meat production using Hanwoo muscle satellite cells has not been reported yet. Hanwoo is an indigenous Taurine cattle in Korea. It is characterized by marbling, soft texture, juiciness, and unique flavor (Lee et al., 2014). Domestic consumption of Hanwoo per capita in 2021 was 4.4 kg, an increase of 4.8% compared to 2020 when it was 4.2 kg, showing a continuous increase in Hanwoo consumption (Hanwoo Association, 2022). As such, we judge that it would be good to use Hanwoo, which accounts for a large portion of domestic beef consumption, as a key raw material for cultured meat research, like other breeds of cattle abroad where research is actively underway for cultured meat production.

Cultured meat shows a lot of potential as a solution to various problems, including environmental pollution, methane emission, land and water shortage, and the welfare of livestock caused by consumption of meat food (Bhat et al., 2011). Despite many advantages of cultured meat, the high production cost compared to meat produced by a general method is the reason for the lack of its popular consumption (Choi & Shin, 2019). To lower the production cost of cultured meat, culture efficiency should be good above all else. Thus, it is necessary to study a method to increase the culture efficiency by shortening the time for proliferation and differentiation of cells to prepare cultured meat. Cell culture may vary depending on various external conditions such as cell type, compositions of culture medium, pH of culture, time of culture, air conditioning, growth factors, mechanical, electronic, gravitational, and so on (Edelman et al., 2005; Simsa et al., 2019). Among which various external conditions, satellite cells are very sensitive to temperature changes, which can greatly affect their proliferation and differentiation (Halevy et al., 2000). For example, in the case of primary human skeletal muscle cultured cells, human skeletal muscle myoblasts, and C2C12 mouse myoblasts, diameters of myotubes cultured at 39 ℃ are larger than those cultured at 37 ℃ (Yamaguchi et al., 2010). In addition, cells cultured at 43 ℃ could not proliferate well due to heat shock (Bolus et al., 2018). When cells are exposed to a high temperature above a certain level, various cellular dysfunctions such as inhibition of protein synthesis, defects in protein structure and function, and changes in morphology due to cytoskeletal rearrangement can occur (Sonnal et al., 2002).

To provide data for using Hanwoo muscle satellite cells as cultured meat, the proliferation and differentiation efficiency of Hanwoo cells in comparison with C2C12 cells according to culture temperature were determined.

2. Materials And Methods

2.1. Cells

Hanwoo satellite cells used in the experiment were collected from the round top muscle of 34-month-old Hanwoo at Farmstory Hannaeng located in Eumseong-gun, Chungcheongbuk-do, Korea. These collected muscles were moved to the laboratory using an ice box. Hanwoo muscle satellite cells were collected with an isolation technique in the laboratory. C2C12 cells (ATCC® CRL-1772™) are commonly used in cultured meat experiments. Thus, they were compared with Hanwoo cells in the present study.

2.2. Cell Proliferation and Differentiation

A flask coated with collagen for proliferation and a flask coated with Matrigel for differentiation were prepared. The collagen coating solution was prepared by diluting a collagen solution of 5 mg/mL with distilled water to a concentration of 0.5%. 1M acetic acid was added to the collagen coating solution to have a final concentration of 2% for collagen solubilization. After dispensing the prepared collagen solution into a flask, it was left in an incubator at 37 ℃ for at least 16 hours. After removing the coating solution by suction before using in the experiment, the flask was washed twice using 1X phosphate buffered saline (PBS). It was then dried for use in the experiment. For Matrigel coating, a solution of Matrigel was prepared by diluting it with cold 1X PBS at a ratio of 1:200. After Matrigel coating, the flask was then left in an incubator at 37 ℃ for at least 4 hours. After removing the coating solution using suction, the flask was washed once with 1X PBS. It was then dried before it was used in the experiment.

For cell proliferation, Ham's F-10 (11550-043, Gibco, USA) medium supplemented with 20% fetal bovine serum (16000-044, Gibco, USA) and 1% penicillin-streptomycin-amphotericin B (PSA) mixture (17-745E, Lonza, USA) was used. For effective cell proliferation and growth, basic fibroblast growth factor (bFGF) at a final concentration of 0.05% was added to the flask. Medium used for differentiation was DMEM (11995-065, Gibco, USA) supplemented with 2% FBS and 1% PSA. For cell proliferation during experiments, Hanwoo cells and C2C12 cells were seeded at a density of 1,800 cells/cm^2 and cultured in an incubator at 37 ℃ with 5% CO_2 nor 39 ℃ with 5% CO_2 for 5 days and 4 days, respectively. In the case of differentiation, cells were cultured in growth medium (GM) until confluent in a 96-well plate or a T25 flask and then differentiated into muscle myotubes in differentiation medium (DM).

2.3. Cell counting

Cells were detached with trypsin. The number of cells was counted using a cell counter (Countess® cell FL automated cell counter, Invitrogen, USA) after staining with trypan blue. A trypsin neutralization solution (2% FBS in PBS) was used to neutralize trypsin.

2.4. Immunofluorescence staining

To measure proliferative capacity using an antibody, Hanwoo cells and C2C12 cells were seeded at a density of 1,800 cells/cm^2 in a 96-well plate and cultured in an incubator at 37 ℃ with 5% CO_2 or 39 ℃ with 5% CO_2 for 3 days and 4 days, respectively. After removing the culture medium, cells were washed with 1X PBS and treated with 2% paraformaldehyde (in PBS) at 37° C for 45 minutes. After washing twice with 1X PBS, cells were treated with 0.1% Triton X (in PBS) at room temperature for 20 minutes. Thereafter, cells were incubated with 2% BSA at room temperature for 30 minutes, washed twice with 1X

PBS, and incubated with Pax7 antibody at 4 ℃ overnight. After washing twice with 1X PBS, cells were incubated with secondary antibody (Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488) at room temperature for 2 hours. Finally Hoechst reagent was added. Immunofluorescence-stained Hanwoo muscle satellite cells and C2C12 cells were observed with an EVOS-5000 optical microscope. Images were obtained after dividing each well into five zones. Each treatment was repeated in five wells. The number of muscle cell nuclei was counted by an experienced expert on imaging data using ImageJ program.

To measure the differentiation, Hanwoo muscle satellite cells and C2C12 cells were seeded into 96-well plates coated with Matrigel at 1,800/cm^2 each well and then cultured at 37°C or 39°C. After proliferation for 3 days and 4 days, respectively, DM was used to replace growth medium. Differentiation was performed for 4 days and 5 days, respectively. Differentiation ability was measured using an antibody with same procedure as described for measuring proliferation except that a monoclonal anti-myosin antibody was used as the antibody and goat anti-mouse IgG1 cross-adsorbed secondary antibody, Alexa Fluor 488 was used as the secondary antibody.

2.5. Western Blotting

Hanwoo muscle satellite cells and C2C12 cells were dispensed into Matrigel pre-coated T25 flasks and cultured at 37 ℃ and 39 ℃ as described above. When these T25 flasks were more than 80% confluent, GM was replaced by DM. Differentiated Hanwoo muscle cells and C2C12 cells in T25 flasks were sampled for western blotting. These T25 flasks were kept cold and washed with cold 1X tris-buffered saline (TBS). Then 1X radio-immunoprecipitation assay (RIPA) lysis buffer was dispensed and cells were separated from the flask using a cell scraper. Protein concentrations in samples were measured by Bradford assay. These quantitatively measured protein samples were separated by TGX Precast Gel (Biorad) and transferred to an ImmunBlot PVDF membrane. The membrane was blocked with EveryBlot Blocking Buffer at room temperature for 1 hour. Proteins of Hanwoo muscle satellite cells were then incubated with primary antibodies against β-actin, myosin, and cytochrome C levels for one day at 4 °C. Proteins of C2C12 cells were incubated with primary antibodies against β-actin and myosin at 4 ℃ for one day. Membranes were washed twice in tris buffered saline with tween® 20 (TBST) at room temperature for 10 minutes and then incubated with Affinity Purified Goat Anti-Mouse IgG (H + L) HRP-conjugated antibody at room temperature for 1 hour. After washing 4 times with TBST again (10 minute each wash), Clarity western ECL substrate (Bio-Rad) was used to detect proteins.

2.6. Reverse transcription and quantitative PCR

Hanwoo muscle satellite cells and C2C12 cells were dispensed into Matrigel pre-coated T25 flasks and cultured at 37 ℃ and 39 ℃ in the same manner as described above. When the T25 flask was more than 80% confluent, GM was replaced by DM. Muscle stem cell samples were collected by scraping differentiated Hanwoo muscle cells into a T25 flask with a cell scraper. TRIzol reagent was used for RNA extraction. In the case of Hanwoo muscle satellite cells, expression levels of myogenin, MyHC (myosin heavy chain), MRF4 (as genes related to muscle formation) and myoglobin (a gene related to maturation)

were determined. cDNA was prepared using a Reverse Transcription Master Premix (ELPIS-BIOTECH, Korea). For RT-qPCR of Hanwoo muscle satellite cells, a template RNA Primer Mixture was prepared using 1.0 µg of mRNA as a template according to the manufacturer's manual, followed by incubation at 60 ℃ for 60 minutes and 94 ℃ for 5 minutes. Expression levels of myogenin, MyHC, MRF4, and myoglobin were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). *Gapdh* gene was used as an internal control for expression level analysis. The qRT-PCR reaction had a volume of 20 µl consisting of 1 µl of cDNA as a template, 10 µl EzAmp™ FAST qPCR 2X Master Mix (ELPIS - BIOTECH, Korea), and 1 µl of each primer. Amplification was conducted at 95 ℃ for 10 minutes followed by 40 cycles of 95 ℃ for 10 seconds and 60 ℃ for 20 seconds. Gene-specific primers were: *myogenin*, Forward – AGA AGG TGA ATG AAG CCT TCG A and Reverse – GCA GGC GCT CTA TGT ACT GGA T; *MyHC*, Forward - AGG AAG AGT TCC AGA AAA CC and Reverse - TGG AGC TGT AGG TCA TTT TT; *MRF4*, Forward - GGT GGA CCC CTT CAG CTA CAG and Reverse - TGC TTG TCC CTC CTT CCT TGG; *myoglobin*, Forward - AAG AGG TGG ATG GGT TAG GG and Reverse - GGC ATT GAG GTG AAA GGA AA; and *Gapdh*, Forward - CAC CCT CAA GAT TGT CAG C and Reverse - TAA GTC CCT CCA CGA TGC.

RT-qPCR of C2C12 cells was the same as that of Hanwoo muscle satellite cells except that myogenin, MyHC, and myoglobin expression levels were measured. Amplification was conducted at 95 ℃ for 10 minutes followed by 40 cycles of 95 ℃ for 10 seconds and 53 ℃ for 20 seconds. Gene-specific primers were: myogenin, Forward – GCC CAG TGA ATG CAA CTC CCA CA and Reverse – CAG CCG CGA GCA AAT GAT CTC CT; MyHC, Forward - AGC AGC GAC ACT GAA ATG GA and Reverse - GTT GTC GTT CCT CAC GGT CT; myoglobin, Forward - GGA AGT CCT CAT CGG TCT GT and Reverse - GCC CTT CAT ATC TTC CTC TGA; and Gapdh, Forward - GTG GCA AAG TGG AGA TTG TTG CC and Reverse - GAT GAT GAC CCG TTT GGC TCC.

2.7. Statistical Analysis

All statistical analyses including Student's t-test were carried out using SAS Statistical Package 9.4 (SAS, 2003). P-values < 0.05 indicated significant differences.

3. Results And Discussion

3.1. Effect of Culture Temperature on Viability and Growth of Hanwoo Cells and C2C12 Cells

Hanwoo satellite cells and C2C12 cells in flasks were cultured at 37 ℃ or 39 ℃ for 4 days and 5 days (Fig. 1a-a‴), respectively. Live cell count and viability were measured. Doubling time was calculated to compare differences by culture temperature. For both Hanwoo cells and C2C12 cells, more live cells were obtained at 37 ℃ than at 39 ℃ based on live cell count. Cell viability was also found to be higher at 37 ℃ than at 39 ℃ (Fig. 1c-e). Heat stress is known to negatively affect skeletal muscle growth by converting energy and nutrients to body temperature maintenance. Acute heat stress has been found to affect cell function by increasing proteolysis and reducing protein synthesis (Febbraio, 2001). Basavaraj et al. (2019) have shown that when C2C12 cells are cultured at 37 ℃ or 39 ℃, total protein content of C2C12 cells cultured at 39 ℃ is lower than that of cells cultured at 37 ℃. In addition, the present study

found that values of maximum resolution (MR) and spare resolution capacity (SRC) as indicators of mitochondrial function were significantly reduced in cells cultured at 39 ℃ than in cells cultured at 37 ℃, indicating that 39 ℃ caused damage to C2C12 cells.

Hanwoo cells and C2C12 cells confluent in flask were differentiated with differentiation medium. It was confirmed that Hanwoo cells differentiated at 37 ℃ had root canal formation after culturing for 2 days and 3 days. They were highly differentiated after culturing for 4 days and 5 days. The root canal tore off after culturing for 6 days. On the other hand, Hanwoo cells cultured at 39 ℃ were differentiated after 1 day of culture. They were highly differentiated after culturing for 2 days and 3 days. The root canal quickly tore off after culturing for 4 days. In the case of C2C12 cells, it was confirmed that root canal was formed after culturing for 3 days. The root canal was highly differentiated after culturing for 5 days. It tore off after culturing for 7 days. C2C12 cells cultured at 39 ℃ showed differentiation after 2 days. The root canal was highly differentiated after culturing for 4 days. It tore off after culturing for 7 days (Fig. 1b-b‴).

3.2. Immunofluorescence Staining

Paired box 7 (Pax7), a transcription factor that controls the proliferation and differentiation of satellite cells during muscle formation, is an essential factor for muscle formation and differentiation of satellite cells into myoblasts (Maltzahn et al., 2013). Hoechst dye is the most popular dye that can distinguish cell nuclei due to their high affinity and specificity for DNA. To indirectly compare proliferative power of cultured Hanwoo cells and C2C12 cells, immunofluorescence staining was performed to observe cells and cell nuclei using Pax7 antibodies and Hoechst. When immunofluorescence staining was performed for Hanwoo cells cultured for 3 days using Pax7 antibodies and Hoechst, the blue color indicated nucleus and the green color indicated Pax7 (Fig. 2a-d”). When the number of nuclei was calculated based on stained cells and nuclei, the distribution of Hanwoo cells cultured at 37 ℃ for 3 days appeared to be significantly higher ($p < 0.05$) that that cultured at 39 ℃. Distribution of C2C12 cells cultured at 37 ℃ for 4 days was also higher than that cultured at 39 ℃ ($p < 0.05$) (Fig. 2e).

Immunofluorescence staining was performed using myosin antibodies and Hoechst to indirectly compare differentiation power of differentiated Hanwoo cells and C2C12 cells. Myosin is a representative structural protein of muscle cells and functional protein that enables muscle contraction and relaxation as the main component of the myosin filament of root fiber. It is judged as an appropriate marker for the degree of cell differentiation (SIN et al., 1986). When dyed area was observed under a microscope, the blue color indicated the nucleus and the green color indicated myosin (Fig. 3a-d”). When immunofluorescence staining was performed to calculate root width, number of nuclei, and fusion index, it was found that Hanwoo cells differentiated at 39 ℃ and 37 ℃, showed no significant difference in root thickness and fusion index of cells (Fig. 3f, g). However, cells differentiated at 37 ℃ had significantly higher ($p < 0.05$) number of nuclei (Fig. 3e). In addition, C2C12 cells differentiated for 4 days at 39 ℃ had a slightly higher number of nuclei that those differentiated for 4 days at 37 ℃ ($p < 0.05$) (Fig. 3e),

although there was no significant difference in the thickness of differentiated root canal and fusion index between cells cultured at 39 ℃ and those cultured at 37 ℃ (Fig. 3f, g).

3.3. Western Blot

Western blot was performed to determine myosin and cytochrome C levels in Hanwoo cells differentiated for 3 days at 37 ℃ or 39 ℃ (Fig. 4a-d'). Cytochrome C, a water-soluble peripheral membrane protein, is known to be an essential component of mitochondrial respiration (Boyer et al., 1977). Protein levels was measured to determine the degree of differentiation into root canals. When western blot results of Hanwoo cells differentiated at 37 ℃ and 39 ℃ were compared, levels of myosin and cytochrome C protein in Hanwoo cells differentiated at 39 ℃ were measured to be relatively higher than those at 37 ℃, although such differences were not statistically significant (Fig. 4e, f). Levels of myosin in C2C12 cells differentiated at 39 ℃ for 3 days were also measured to be relatively higher than those at 37 ℃, although there was no significant difference in the relative fluorescence intensity of the band (Fig. 4g). A similar pattern was found for myotubes based on immunofluorescence staining. To confirm the difference in protein expression levels for cells cultured at 37 ℃ and 39 ℃, it is necessary to proceed with additional experiments using myosin and cytochrome C as well as other protein antibodies. According to Kanatous & Mammen (2010), high levels of actin and myosin are induced by electrical stimulation of C2C12 cells during differentiation. Even in this case, it is necessary to find the factor that can enhance the expression of these proteins. According to Liu & Brooks (2011), expression levels of several genes including transcription factors involved in mitochondrial biogenesis are increased during mild heat shock when the temperature not excessively high, suggesting that there is an additional reason for the absence of cytochrome C protein. Kang (2005) has shown that mild heat stress plays a beneficial role in organisms through facilitating growth factor-mediated cell survival and proliferation. Mild heat stress may act as one physicochemical signal to regulate activities of membrane proteins by affecting membrane fluidity. In additional experiments, C2C12 cells were cultured until passage no. 9 by subculture after proliferating for 4 days. After differentiation through DM for 5 days, western blotting result revealed that myosin protein expression in cells cultured at 39°C was significantly increased ($p < 0.05$) than in cells cultured at 37 °C. It seems that a certain amount of subculture and time are needed for a differentiation to occur in this experiment through a temperature change.

3.4. RT-qPCR

RT-qPCR was performed to confirm quantitative gene amounts of myogenin, myosin heavy chain (MyHC), myogenic factor 4 (MRF4), and myoglobin in Hanwoo cells differentiated for 3 days at 37 ℃ and 39 ℃. Myogenin is one of MRFs. It regulates proliferation and differentiation of satellite cells, precursor cells of myofibers (Kim, 2009). That is, it is expressed in the nucleus of activated satellite cells for the regeneration of myofibers. It affects the process of myofiber growth by regulating the formation of myoblasts and myotubes (Grounds, 1992). MRF4 is a transcription factor that regulates myogenic and amnion cells involved in skeletal muscle formation. It is known to be abundant in adult muscle cells (Yaniv et al., 2010). MyHC is also an important factor in determining muscle contractility. It is a late-stage

marker of muscle cell differentiation. Its expression level increases as muscle cell differentiation proceeds (Park et al., 1999). Myoglobin is a cytoplasmic protein expressed only in cardiac muscle cells and oxidized skeletal muscle fibers (Park et al., 1999). As an oxygen storage protein in muscle, it serves to buffer the concentration of intracellular oxygen and promote intracellular oxygen diffusion when muscle activity increases (Ordway & Garry, 2004). In addition, myoglobin is a meat pigment that determines the red color of meat. It has a heme ring containing iron atoms. Thus, when we eat meat, we feel a quick taste of blood (Yancey et al., 2006). Looking at the flow of differentiation, when Pax7 and Pax3 are expressed in satellite cells, they express Myf5 and MyoD and differentiate into dividing myoblasts. These myoblasts are differentiated into myocytes while expressing myogenin and MRF4. Then myocytes stop dividing and form multinucleated myotubes (Ministry of Health and Welfare, 2017). When expression levels of the above gene were measured to determine the degree of formation of differentiated myotubes, gene levels of myogenin in cells differentiated at 39 ℃ were higher than those in cells differentiated at 37 °C, although these differences were not statistically significant. Gene levels of MyHC ($p < 0.05$), MRF4, and myoglobin ($p < 0.01$) were significantly higher in cells cultured at 39 ℃ than in cells cultured at 37 ℃ (Fig. 5a). In the case of C2C12, gene levels of MyHC in cells differentiated at 39 ℃ were higher than in cells differentiated at 37 °C. However, such differences were not statistically significant. Relative gene levels of myogenin and myoglobin were significantly higher in cells cultured at 39 ℃ than in cells cultured at 37 ℃ ($p < 0.05$) (Fig. 5b). MyHC, MRF4, and myoglobin gene levels in Korean beef muscle satellite cells and myogenin and myoglobin gene levels in C2C12 cells were significantly higher in cells cultured at 39 ℃ than in cells cultured at 37 ℃. However, there was no specific difference, meaning that genes involved in differentiation were present but not expressed. According to Zak et al. (2016) and Shi et al. (2016), exposure to a high temperature or a low temperature did not have a significant effect on human skeletal muscle gene expression or affect gene expression of MyHC type in pig back muscles. Thus, temperature can have various effects depending on the temperature difference and the organism affected by the temperature. Previous experimental results have shown that differentiation of C2C12 cells cultured at a high temperature for a long time into root canals can be inhibited by heat shock (Bolus et al., 2018). In the case of a mild heat stress at a low temperature, cell proliferation, differentiation, and immunity can respond positively (Park et al., 2005). In the case of myocytes of quail, differentiation at 39 ℃ for a long period of time can increase the length and diameter of myotubes than incubation at 37 ℃. Protein contents of slow myosin heavy chain isoform and cytochrome C oxidase subunit IV are higher than others (Chli et al., 2016). In addition, there are experimental results showing that the amount of PGC-1α protein varies according to the duration of heat stress and the temperature (Yamaguchi et al., 2009). As an additional experiment, it was judged that it was necessary to extend the culture period of Hanwoo muscle satellite cells and C2C12 cells through subculture, considering the fact that root canals were thick and plenty in numbers. In addition, the amount of protein was increased when C2C12 cells were subcultured to passage number 9. In addition, heat shock protein (HSP), a defense mechanism to protect itself from external stress (Ellis, 1987), is an important cellular temperature resistance protein to prevent denaturation of polypeptides under heat stress. When cells face stress, intracellular material transport and protein misfolding will occur. HSP can prevent these phenomena, increasing cell viability and helping cells overcome heat stress (Mishra & Palai, 2014). The fact that expression of a specific protein was not

observed, but was expressed after some subculture, was presumed to be related to HSP. A more accurate analysis will be possible if HSP expression level according to temperature and incubation time is checked.

4. Conclusions

Hanwoo muscle satellite cells were cultured at 37 ℃ and 39 ℃ to compare myoblast cells, myotube cells, and C2C12 cells widely used for cultured meat production. When live cell count, cell viability, and doubling time of Hanwoo muscle satellite cells and C2C12 cells cultured at 37 ℃ and 39 ℃ were compared, both cells proliferated at 37 ℃ showed more proliferation than those proliferated at 39 °C. In addition, as a result of confirming Pax7 and Hoechst of proliferated cells using immunofluorescence staining, it was found that Hanwoo muscle satellite cells and C2C12 cells cultured at 37 ℃ proliferated more than those cultured at 39 °C. Immunofluorescence staining, western blot, RT-qPCR, and DNA electrophoresis were used to measure cell differentiation. Immunofluorescence staining of differentiated cells with myosin and Hoechst revealed that the number of nuclei was higher in cells differentiated at 37 ℃ than in cells differentiated at 39 °C, although the difference was not statistically significant. However, the fusion index was significantly higher at 39 ℃ than that at 37 °C. Western blot experiment revealed that Hanwoo muscle satellite cells differentiated at 39 ℃ had higher myosin and cytochrome C protein levels than those differentiated at 37 C, although these differences were not statistically significant. C2C12 cells differentiated at 39 ℃ also had higher myosin protein levels than those differentiated at 37 °C, although the difference was insignificant. As a result of RT-qPCR experiment, expression levels of MyHC, MRF4, and myoglobin genes in Hanwoo muscle satellite cells differentiated at 39 ℃ were higher than in cells differentiated at 37 °C. Myogenin also had higher gene levels at 39 ℃ than at 37 C. However, the difference was not statistically significant. Myogenin and myoglobin gene levels in C2C12 cells differentiated at 39 ℃ were higher than those at 37 °C. MyHC gene levels at 39 ℃ were also higher than those at 37 °C, but not significantly higher. As a result of confirming gene levels through the band of Hanwoo muscle satellite cells by DNA electrophoresis, MyHC, my oglobin, and MRF4 showed a similar trend to RT-qPCR results. The difference was further reduced. Through additional experiments, when C2C12 cells were proliferated to Passage no. 9 through subculture and then differentiated, it was confirmed that a lot of protein expression. If HSP is analyzed according to the number of passages, it is thought that an accurate cause analysis will be possible.

As a result of analyzing the proliferation and differentiation of Hanwoo muscle satellite cells and C2C12 cells, it was found that the most efficient method was to proliferate both types of cells for a certain passage in a culture environment at 37 ℃ and then differentiate them in a culture environment at 39 ℃. In addition, the pattern of proliferation and differentiation of C2C12 cells used as cultured meat showed a tendency similar to that of Hanwoo satellite muscle cells. The above experimental results can be used as reference data to utilize Hanwoo muscle satellite cells for cultured meat production.

5. Declarations

5. Compliance with Ethical Standards

5.1. Conflict of Interests

The authors declare no conflicts of interest.

5.2. Funding

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5.3. Ethical Approval

The animal study protocol was approved by Chungbuk National University Institutional Animal Care and Use Committees.

5.4. Informed Consent

Informed consent was obtained from all subjects involved in the study.

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Figures

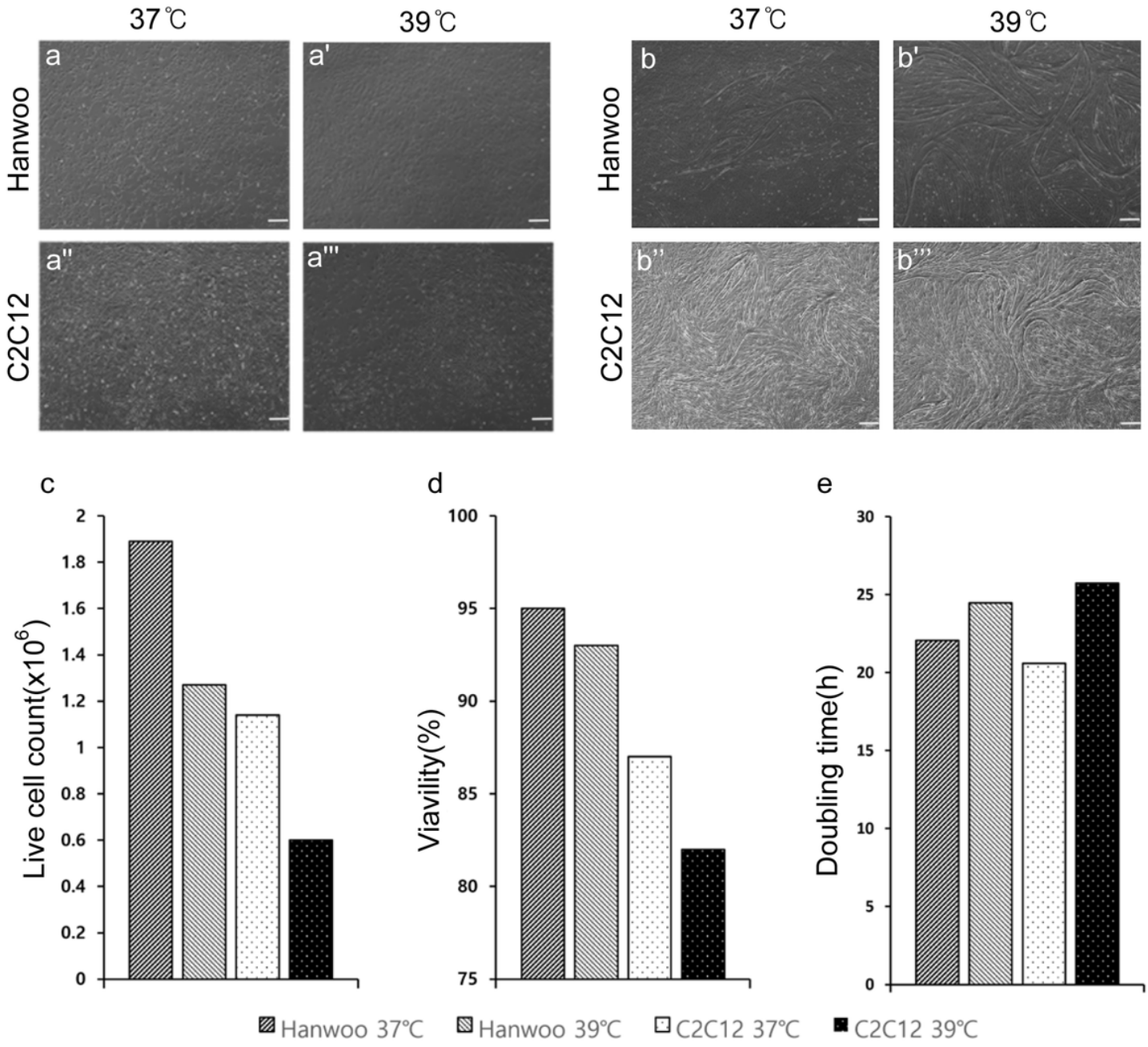

Figure 1

Live cell count, cell viability and doubling time of Hanwoo satellite cells and C2C12 cells proliferated and differentiated in flasks at 37 ℃ and 39 ℃. a-a‴: Hanwoo satellite cells and C2C12 cells proliferated for 4 days and 5 days, respectively, at 37 ℃ or 39 ℃. b-b‴: Hanwoo cells and C2C12 cells differentiated for 3 days at 37 ℃ or 39 ℃. c-e: Live cell count, viability, and doubling time of Hanwoo cells and C2C12 cells

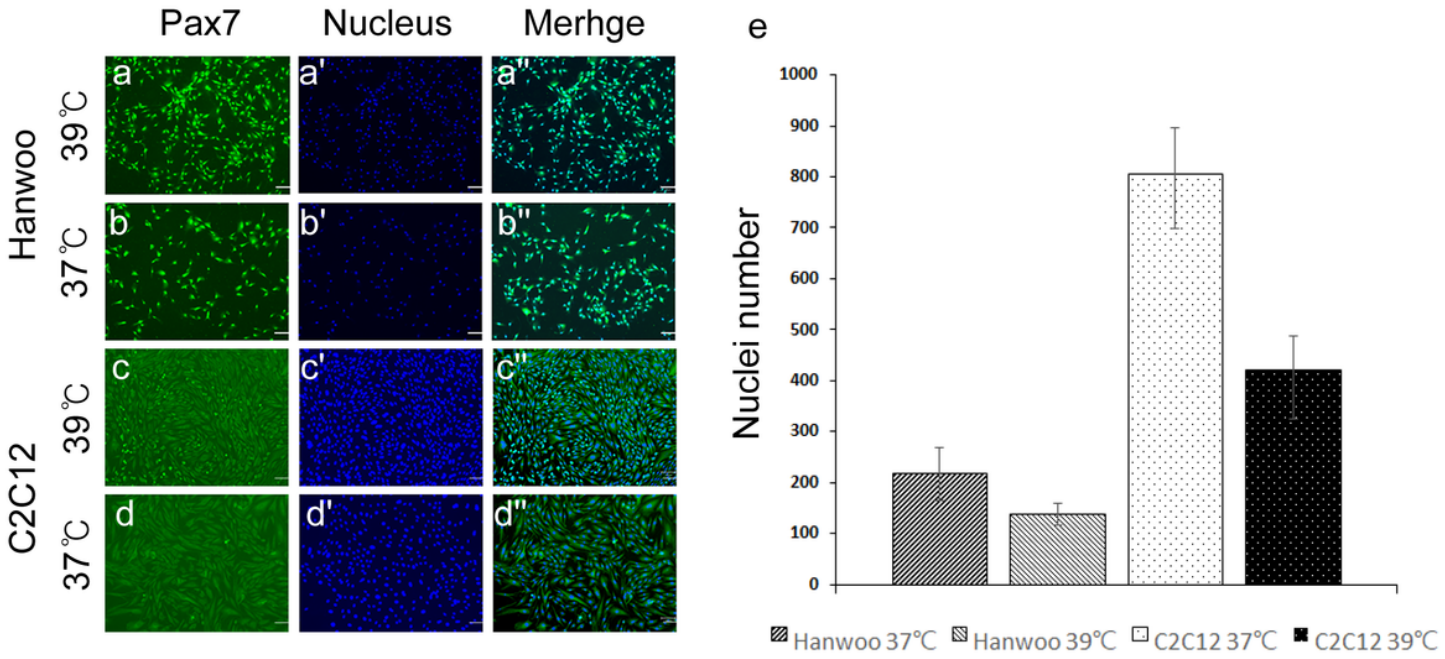

Figure 2

Immunofluorescence staining was performed to observe cells and cell nuclei of proliferated Hanwoo cells and C2C12 cells using Pax7 antibodies and Hoechst. a-d‴: Immunofluorescence staining was performed for Hanwoo cells and C2C12 cells cultured for 3 days using Pax7 antibody and Hoechst. The blue color indicates nucleus and the green color indicates Pax7 (scale bar: 100 μm). e: Nuclei numbers of Hanwoo cells and C2C12 cells were counted using ImageJ

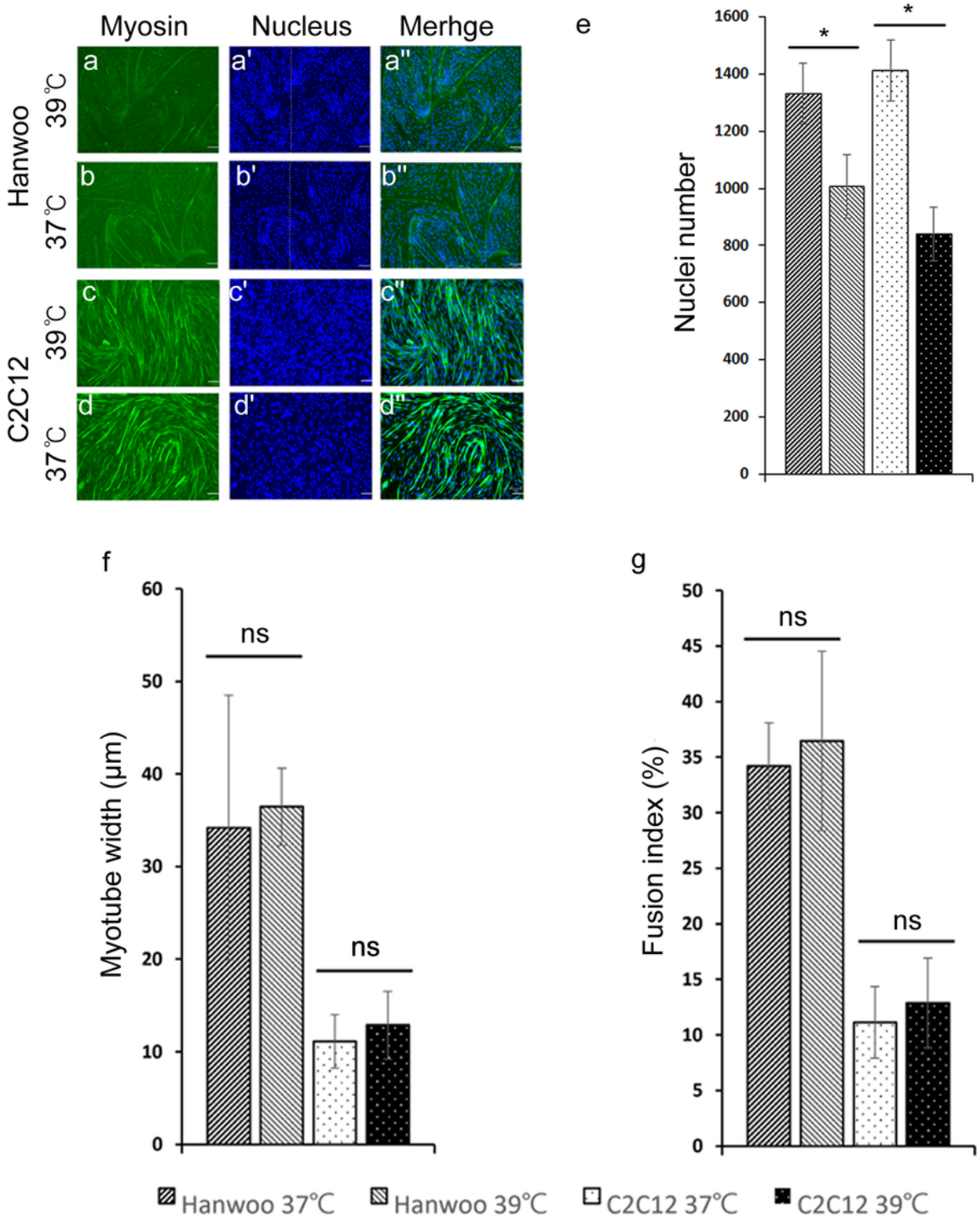

Figure 3

Immunofluorescence staining was performed to observe cells and cell nuclei of differentiated Hanwoo cells and C2C12 cells using myosin antibodies and Hoechst. a-d”: Immunofluorescence staining was performed for Hanwoo cells and C2C12 cells differentiated for 3 days using Pax7 antibody and Hoechst. The blue color indicates nucleus and the green color indicates Pax7 (scale bar: 100 μm). e-g: Nuclei

numbers, myotube width, and fusion index of Hanwoo cells and C2C12 cells were measured using ImageJ (*: $p < 0.05$)

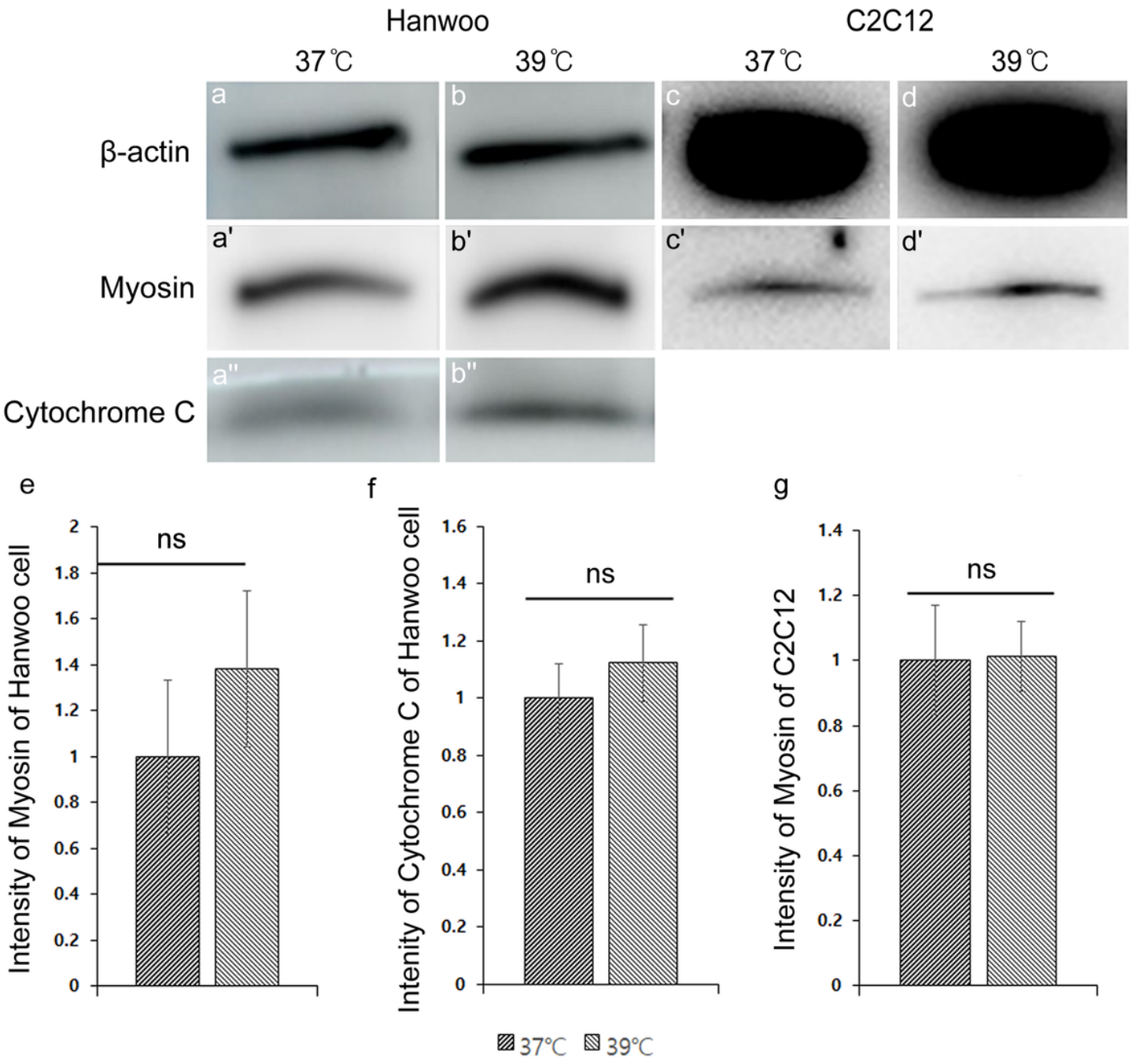

Figure 4

Expression levels of myosin and cytochrome C proteins in Hanwoo cells and C2C12 cells measured by Western blotting. a-d': Western blot results of Hanwoo cells differentiated at 37 ℃ and 39 ℃ were confirmed through band intensity. e, f: Intensities of myosin and cytochrome C bands in Hanwoo cells differentiated at 37 ℃ and 39 ℃ in Western blotting were measured. g: Intensities of myosin in C2C12 cells differentiated at 37 ℃ and 39 ℃ in Western blotting were measured

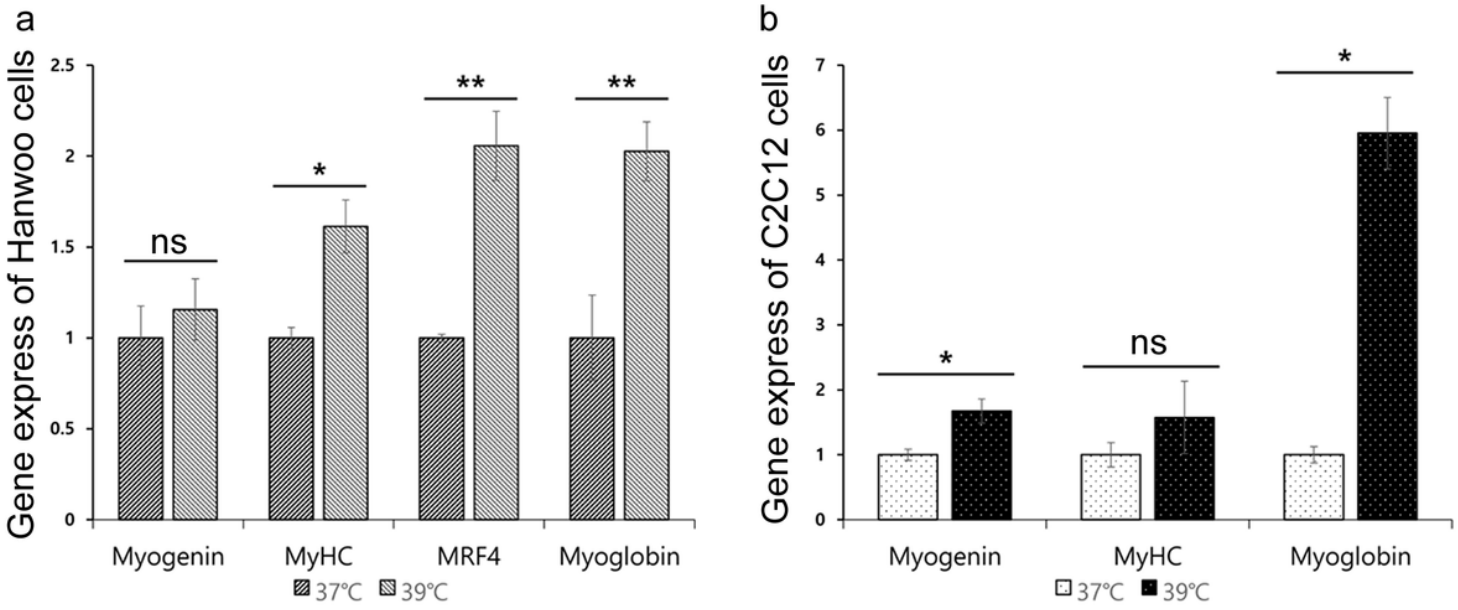

Figure 5

Gene levels of myogenin, MyHC, MRF4, and Myoglobin in Hanwoo cells and C2C12 cells were measured using RT-qPCR. a: Expression levels of myogenin, MyHC, MRF4 and myoglobin genes in Hanwoo cells differentiated at 37 ℃ and 39 ℃ were measured using RT-qPCR (*: $p < 0.05$, **: $p < 0.01$). b: Expression levels of myogenin, MyHC, and myoglobin in C2C12 cells differentiated at 37 ℃ and 39 ℃ were measured using RT-qPCR (*: $p < 0.05$)