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# Developmental ability of Hanwoo muscle satellite cells under culture conditions mimicking the *in vivo* environment

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Cultivated meat refers to edible meat obtained by proliferating cells without killing livestock in a laboratory. The selection of donor animals is a crucial factor for efficient cell culture production. Hanwoo is a native Korean taurine cattle breed raised as livestock in Korea since before 2000 B.C. Cells isolated from Hanwoo, which has little genetic diversity, are expected to be advantageous in cell culture because of the existence of fewer individual differences. However, cells collected from Hanwoo are in a state where efficient culture conditions have not been established. Therefore, in this study, we investigated the effects of mimicking an *in vivo* environment on the proliferation and differentiation of Hanwoo muscle satellite cells. The culture conditions consisted of CON (37°C/20% O<sub>2</sub>), T1 (37°C/2% O<sub>2</sub>), T2 (39°C/20% O<sub>2</sub>), and T3 (39°C/2% O<sub>2</sub>). Cell numbers decreased and expression levels of PAX7 and MYF5 decreased at a temperature of 39°C ( $p < 0.05$ ). Conversely, 2% oxygen increased the number of cells and expression levels of PAX7 and MYF5 ( $p < 0.05$ ). A temperature of 39°C inhibited the proliferation of Hanwoo muscle satellite cells by reducing the expression of PAX7 and MYF5 ( $p < 0.05$ ). Conversely, 2% oxygen promoted the proliferation of Hanwoo muscle satellite cells by enhancing the expression of PAX7 and MYF5 ( $p < 0.05$ ). During differentiation, a temperature of 39°C improved the myotube area and fusion index ( $p < 0.05$ ). The RT-qPCR and Western blotting results revealed that a culture temperature of 39°C increased expression levels of the MYH2 gene and DES and MYOG proteins ( $p < 0.05$ ). Additionally, an interactive condition increased expression levels of MYOD1, DES, and MYOG genes ( $p < 0.05$ ). These results indicated that a temperature of 39°C promoted the differentiation of Hanwoo muscle satellite cells by increasing DES and MYOG protein expression. Thus, the production of cultivated meat using Hanwoo muscle satellite cells is expected to be efficient under 2% oxygen for proliferation and 39°C for differentiation.

## KEYWORDS

cultivated meat, culture condition, differentiation, Hanwoo, *in vivo* environment, muscle satellite cells, proliferation

## 1 Introduction

The consumption of meat has long been considered a vital source of essential nutrients in the human diet and an indicator of societal development (Ahmad et al., 2018; Van der Weele et al., 2019). The supply of foods such as meat needs to be increased from limited resources to meet the food requirements of the world's growing population (Godfray et al., 2018; Chriki

and Hocquette, 2020). Global meat demand has tripled over the past 50 years, reaching 340 million tons in 2018 (Chen et al., 2022). However, conventionally produced meat is one of the most intensive land uses per kilogram of protein and a major source of greenhouse gases (Ritchie et al., 2022). Therefore, efficient protein production methods are being developed. Cellular agriculture is a technology in which meat and other agricultural products are produced by culturing cells rather than rearing livestock (Mattick, 2018). Cultivated meat refers to edible meat obtained by proliferating cells without animal slaughter. It is also called cell-based meat, clean meat, or *in vitro* meat (Allan et al., 2019; Lee et al., 2021; Treich, 2021). During cultivated meat production, cells are harvested through a biopsy conducted on a host animal (cattle, pig, and sheep), followed by their proliferation, differentiation, and production into meat tissues (Humbird, 2021; Lanzoni et al., 2022). Various factors, such as age, gender, and breed of the donor, can affect the production quantity and quality of cells (Melzener et al., 2022). Therefore, the selection of donor animals is a crucial factor for efficient cell culture production (Martins et al., 2023). Hanwoo is a native Korean taurine cattle breed raised as livestock in Korea since before 2000 B.C. (Jo et al., 2012; Lee et al., 2014). Korea has four varieties of Hanwoo cattle: brown (Major Hanwoo), black face (Heukwoo), black (Jeju Heukwoo), and tiger color (Chickso). Among them, Brown Hanwoo is the most common type of cattle being raised (Jo et al., 2012). Hanwoo has a low milk production capacity and a slow growth rate. However, it has excellent reproductive ability and relatively good meat quality (Kim and Lee, 2000). Hanwoo is mainly isolated within the Korean Peninsula. Its lineage is managed through pure breeding. Therefore, its genetic diversity is lower than that of other species (Joo et al., 2017). Cells isolated from Hanwoo, which has little genetic diversity, are expected to be advantageous in cell culture because of the existence of fewer individual differences (Park et al., 2023). However, cells collected from Hanwoo are in a state where efficient culture conditions have not been established yet. In general, the core temperature of Hanwoo is 38.5°C, and physiological oxygen concentrations in its muscles are approximately 2.3 to 6.0% (Ast and Mootha, 2019; Kim et al., 2020). Therefore, in this study, we investigated the effects of culture conditions mimicking the *in vivo* environment of Hanwoo on the proliferation and differentiation of Hanwoo muscle satellite cells.

## 2 Materials and methods

### 2.1 Isolation of Hanwoo muscle

The *gluteobiceps* muscle tissues of a 25-month-old Hanwoo were collected by removing the surface fat tissue from the hip area and using a scalpel from Dong-A Food Co., Ltd., Heungdeok-gu, Cheongju-si, Chungcheongbuk-do, stored in an icebox, and transported to the laboratory, where connective and adipose tissues were removed. The extracellular matrix (ECM) in muscle tissues was decomposed using collagenase type II mix (Cat. # LS004176, Worthington Biochemical, Columbus, Ohio, USA) and centrifuged alternately at 70 g for 3 min and 800 g for 5 min to isolate the cells. The cell pellet was obtained through centrifugation and filtration through a 100- $\mu$ m cell strainer (Cat. # 93100, Cytiva, Marlborough, MA, USA) and a 40- $\mu$ m strainer (Cat. # 431750, Corning, Corning, NY, USA). Red blood cells were then lysed with ammonium chloride-potassium

lysis buffer (Cat. # A1049201, Gibco, Waltham, MA, USA). The obtained Hanwoo muscle cells were stored in a freezing medium (Cat. # 12648010, Gibco) and then in liquid nitrogen until use for subsequent experiments. These cells were considered passage 0 (P0).

### 2.2 Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) was performed for isolating bovine muscle satellite cells according to the method of Melzener et al. (2022). The P0 cells were cultured in a collagen-coated flask and then transferred to a FACS buffer (1:100, BSA in PBS). These cells were then incubated with FITC anti-sheep CD31 (1:10, Cat. # MCA1097F, Bio-Rad, Hercules, CA, USA), FITC anti-sheep CD45 (1:10, Cat. # MCA2220F, Bio-Rad), APC anti-human CD29 antibody (1:10, Cat. # 303008, BioLegend, San Diego, CA, USA), and PE-Cy<sup>TM</sup>7 anti-human CD56 (1:10, Cat. # 335826, Becton Dickinson, Franklin, NJ, USA) for 30 min at 4°C. CD31<sup>-</sup>, CD45<sup>-</sup>, CD29<sup>+</sup>, and CD56<sup>+</sup> cells were sorted using the FACS Aria II Cell Sorter (Becton Dickinson). The obtained muscle satellite cells (Figure 1) were seeded into a growth medium (GM; Ham's F-10 nutrient mix (Cat. # 11550043, Gibco) supplemented with 20% fetal bovine serum (FBS) (Cat. # 35-015-CV, Corning), 1% penicillin-streptomycin-amphotericin B (PSA) mixture (Cat. # 17-745E, Lonza, Basel, Swiss), and 5 ng/mL of human fibroblast growth factor-basic recombinant protein (bFGF, Cat. # 13256-029, Gibco) and proliferated at 37°C in an incubator at 5% CO<sub>2</sub>. The obtained muscle satellite cells were stored in a freezing medium (Cat. # 12648010, Gibco) and liquid nitrogen until use for subsequent experiments.

### 2.3 Cell culture and experimental design

The isolated Hanwoo muscle satellite cells were cultured with the GM in a collagen-coated flask. The cells were seeded at a density of 1,800 cells/cm<sup>2</sup> and cultured in separate incubators under CON conditions (37°C, 20% O<sub>2</sub>), T1 conditions (37°C, 2% O<sub>2</sub>), T2 conditions (39°C, 20% O<sub>2</sub>), and T3 conditions (39°C, 2% O<sub>2</sub>) for 5 days. For differentiation, the cells were seeded at a density of 1,800 cells/cm<sup>2</sup> into a flask coated with Matrigel solution (Cat. # 354234, Corning) and proliferated in a humidified incubator at 37°C with 5% CO<sub>2</sub>. When confluence was reached, the GM was replaced with a differentiation medium (DM; Dulbecco's modified eagle's medium (DMEM) (Cat. # 11995-065, Gibco) supplemented with 2% FBS (Corning) and 1% PSA mixture (Lonza)) and cultured in an incubator under each condition.

### 2.4 Cell counting and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

For cell counting, confluent cells were harvested using trypsin-EDTA (Cat. # 25300-062, Gibco). The number of cells was determined by staining with trypan blue (Cat. # T8154, Sigma-Aldrich, Saint Louis, MO, USA). The MTS assay was performed using an MTS

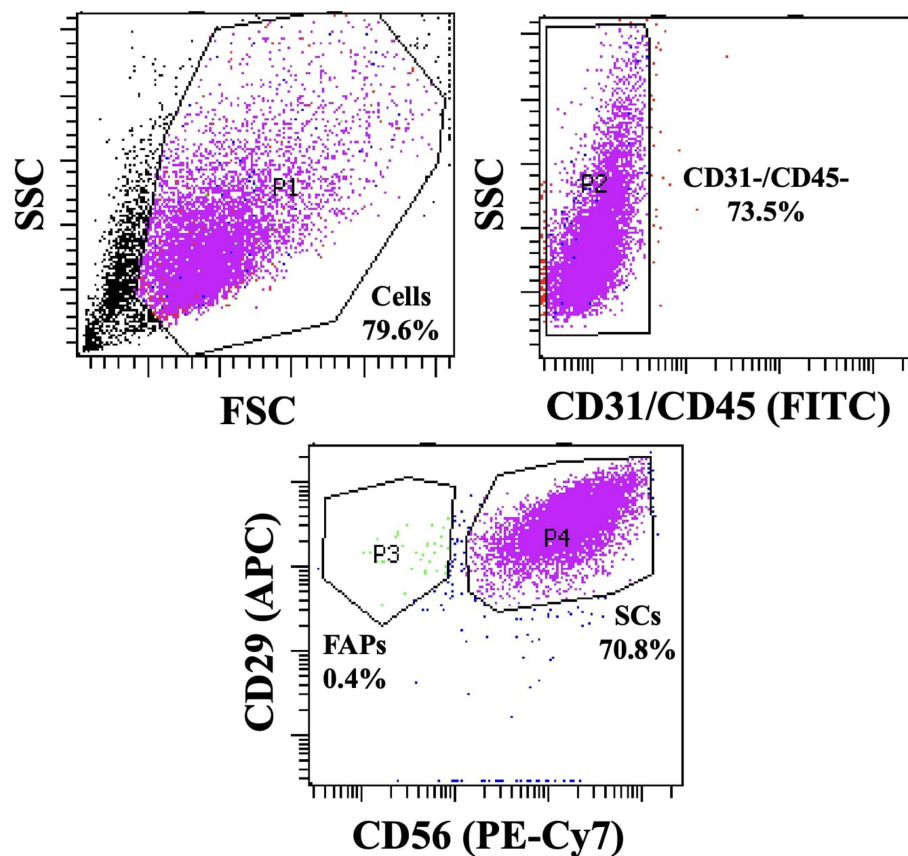


FIGURE 1  
Plot graph of FACS performed using CD31, CD45, CD29, and CD56 antibodies.

reagent (Cat. # G3582, Promega, Madison, WI, USA) in a confluent cell flask. Absorbance was then measured at 490 nm.

## 2.5 Immunofluorescence staining

The Hanwoo muscle satellite cells were fixed with 4% paraformaldehyde (Cat. # SM-P01-050, GeneAll Biotechnology, Seoul, Korea) for 45 min at 37°C. The fixed cells were then permeabilized with 0.1% Triton X-100 (Cat. # T8787, Sigma-Aldrich) and blocked with 2% bovine serum albumin (Cat. # 10738328103, Roche, Basel, Switzerland). The cells were stained with myosin type II antibody (Cat. # M4276, Sigma-Aldrich), secondary antibody (Alexa flour 488; Cat. # A21121, Invitrogen), and Hoechst 33342 (Cat. # H3570, Invitrogen). The image of the stained cells was analyzed using the ImageJ program (NIH).

## 2.6 Reverse transcription and quantitative polymerase chain reaction

The cells were stained with myosin antibody (Cat. # M4276, Sigma-Aldrich), secondary antibody (Alexa flour 488; Cat. # A21121, Invitrogen), and Hoechst 33342 (Cat. # H3570, Invitrogen). The image of the stained cells was analyzed using the ImageJ program (NIH). RNA was extracted from the cells using a Total RNA Extraction Kit

(Cat. # 17221, iNtRON Biotechnology, Seongnam, Korea) and converted to cDNA using a high-capacity cDNA Reverse Transcription Kit (Cat. # 4368814, Applied Biosystems, Waltham, MA, USA). Genes were amplified using a Fast qPCR 2xSYBR Green Master Mix (Cat. # A46012, Applied Biosystems). Proliferation target genes were *PAX7*, *MYOD1*, *MYF5*, and *GAPDH*. Differentiation target genes were *MYH2*, *MYOD1*, *DES*, *MYOG*, *MB*, and *GAPDH*. Table 1 presents primers used for the PCR analysis. Relative gene expression was calculated using the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).

## 2.7 Western blotting

The cells were washed using PBS and lysed on ice using RIPA Lysis Buffer (Cat. # MB-030-0050, Rockland, Philadelphia, PA, USA). The extracted proteins were separated by SDS-PAGE (Cat. # 4561024, Bio-Rad) and transferred to polyvinylidene fluoride (PVDF) membranes (Cat. # 1620175, Bio-Rad). The membranes were blocked with a blocking buffer (Cat. # 120110020, Bio-Rad) and stained with primary antibodies against MYOD1, PAX7, DES, and ACTB. They were then stained with respective affinity-purified goat anti-mouse (H+L)-HRP-conjugated (Cat. # 1706516, Bio-Rad) or affinity-purified goat anti-rabbit IgG (H+L) HRP-conjugated (Cat. # SA002-500, GenDEPOT, Katy, TX, USA) secondary antibodies. The membranes were developed using a Clarity Western ECL Substrate (Cat. # 1705061, Bio-Rad) and visualized on an Amersham™ ImageQuant™ 800 (Cytiva).

TABLE 1 Primer sequences used in RT-qPCR analysis.

Gene	GenBank accession		Primer 5'-3'	Product size (bp)
PAX7	XM_019984705.1	Forward	ATTCCAATGCTAGGACCAAT	225
		Reverse	CTGAGTTGGGTACACACGTT	
MYOD1	NM_001040478.2	Forward	CCTTCCAAGTGTAACAGGT	165
		Reverse	CACCATGCCTCAGAGATAAA	
MYF5	NM_174116.1	Forward	TTGACAGCGTCTACTGTCCT	216
		Reverse	CATGATAGATGAGCCTGGAA	
MYH2	NM_001166227.1	Forward	ATCAGTCAAGGGGAGATCAG	120
		Reverse	GAGCTTGTAGATGGAGACCT	
DES	NM_001081575.1	Forward	GTCAAAGGTGTCTGACCTGA	150
		Reverse	CTCATCAGTGAGTCGTTGGT	
MYOG	NM_001111325.1	Forward	AATCCAGTCCCAAGTCACT	106
		Reverse	GGCAGCTTACAAACAACAC	
MB	NM_173881.2	Forward	AACTTGGGAACCATGAGAAG	183
		Reverse	GATGGGGTCAGAGAATTAGG	
GAPDH	NM_001034034.2	Forward	GTTCGACAGATAGCCGTAAC	138
		Reverse	GATGTCCACTTTGCCAGAAT	

## 2.8 Statistical analysis

All statistical analyses were performed using the SAS program (SAS Institute, Cary, NC, USA). Duncan's multiple range test was used to compare significant differences ( $p < 0.05$ ). A two-way analysis of variance (ANOVA) was used to determine the effects of temperature and oxygen concentration as well as the interaction of these two parameters.

## 3 Results

### 3.1 Proliferation capacity of Hanwoo muscle satellite cells

To investigate the effects of temperature and oxygen concentration conditions on muscle cell proliferation, the Hanwoo muscle satellite cells were grown at different temperatures (37°C and 39°C) and oxygen levels (20 and 2%) for 5 days. Figure 2A presents the image of the Hanwoo muscle satellite cells on day 5 after being grown at different temperatures and oxygen levels. On the fifth day of proliferation, confluence was observed, and cell counting, as well as the MTS assay, was conducted. In addition, the relative mRNA expression levels of PAX7, MYOD1, and MYF5 as crucial muscle-related genes were measured by RT-qPCR. MYOD1 and PAX7 Protein expression levels were measured through Western blotting. The total and live cell numbers were significantly higher in CON and T1 at a temperature of 37°C than in T2 and T3 at a temperature of 39°C ( $p < 0.05$ ) (Figure 2B). MTS absorbance was significantly higher in CON and T1 ( $p < 0.05$ ). Figure 2C illustrates the influence of

each condition and interaction through two-way-ANOVA statistics for all experimental results. The temperature and oxygen conditions affected the total cell number ( $p < 0.0001$ ), live cell number ( $p < 0.0001$ ), and MTS absorbance ( $p < 0.0001$ ). The interactive condition affected the total cell number ( $p < 0.001$ ) and live cell number ( $p < 0.001$ ).

### 3.2 Differentiation capacity of Hanwoo muscle satellite cells

The effects of temperature and oxygen concentration on differentiation were investigated. After the Hanwoo muscle satellite cells became confluent under the same temperature and oxygen conditions, they were differentiated for 2 days at different temperatures (37°C and 39°C) and oxygen levels (20 and 2%). Myotubes were fixed, subjected to immunofluorescence staining for myosin, and counterstained with DAPI (Figure 3A). The plates were imaged, and myogenesis was quantified by measuring the myotube area and fusion index. In addition, the relative mRNA expression levels of MYH2, MYOD1, DES, MYOG, and MB as vital muscle-related genes were measured through RT-qPCR. The DES and MYOG protein expression levels were measured through western blotting. Figure 3B presents the results of the myotube area and fusion index after the differentiation of the Hanwoo muscle satellite cells. The myotube area and fusion index were significantly higher in T2 ( $p < 0.05$ ). The temperature condition affected the myotube area ( $p < 0.05$ ) and the fusion index ( $p < 0.01$ ) (Figure 3C). The oxygen condition affected the myotube area ( $p < 0.01$ ) and the fusion index ( $p < 0.001$ ). The interactive conditions affected the fusion index ( $p < 0.01$ ).

### 3.3 Expression levels of muscle-related genes in the Hanwoo muscle satellite cells

Figure 4 presents the results of relative mRNA expression levels of *PAX7*, *MYOD1*, and *MYF5* genes related to proliferation determined through RT-qPCR. The *PAX7* and *MYF5* expression levels were the highest in T1 and the lowest in T2 ( $p < 0.05$ ). The significantly highest expression level of *MYOD1* was observed in CON, and the lowest expression level was observed in T2 and T3 ( $p < 0.05$ ). The treatment group with the same oxygen conditions and a temperature condition of 39°C exhibited significantly lower expression of all genes than the treatment group with a temperature condition of 37°C ( $p < 0.05$ ). As shown in Figure 4B, the temperature and oxygen conditions significantly affected the expression levels of *PAX7* ( $p < 0.0001$ ), *MYOD1* ( $p < 0.0001$ ), and *MYF5* ( $p < 0.0001$ ). The interactive condition significantly affected *PAX7* ( $p < 0.001$ ), *MYOD1* ( $p < 0.0001$ ), and *MYF5* ( $p < 0.0001$ ). Figure 5 presents the results of relative mRNA expressions of *MYH2*, *MYOD1*, *DES*, *MYOG*, and *MB* related to differentiation by using RT-qPCR. As shown in Figure 5B, the expression of *MYH2* showed the highest value in T2 ( $p < 0.05$ ). In the case of *MYOD1* and *DES*, a significantly higher value was obtained in T3 ( $p < 0.05$ ). The expression of *MYOG* shows a significantly high value in CON and T3 ( $p < 0.05$ ). The expression of *MB* shows the significantly lowest value in CON ( $p < 0.05$ ) with no significant difference ( $p > 0.05$ ) in T1, T2, and T3. Except for *MYH2*, other gene expressions indicated significantly higher values in T3. As shown in Figure 5C, the temperature conditions significantly affected the expressions of *MYH2* ( $p < 0.05$ ), *MYOD1* ( $p < 0.0001$ ), *DES* ( $p < 0.0001$ ), and *MB* ( $p < 0.0001$ ). Oxygen condition significantly affected the expressions of *MYH2* ( $p < 0.0001$ ), *MYOD1* ( $p < 0.0001$ ), *DES* ( $p < 0.0001$ ), *MYOG* ( $p < 0.01$ ), and *MB* ( $p < 0.0001$ ). Interactive conditions significantly affected the

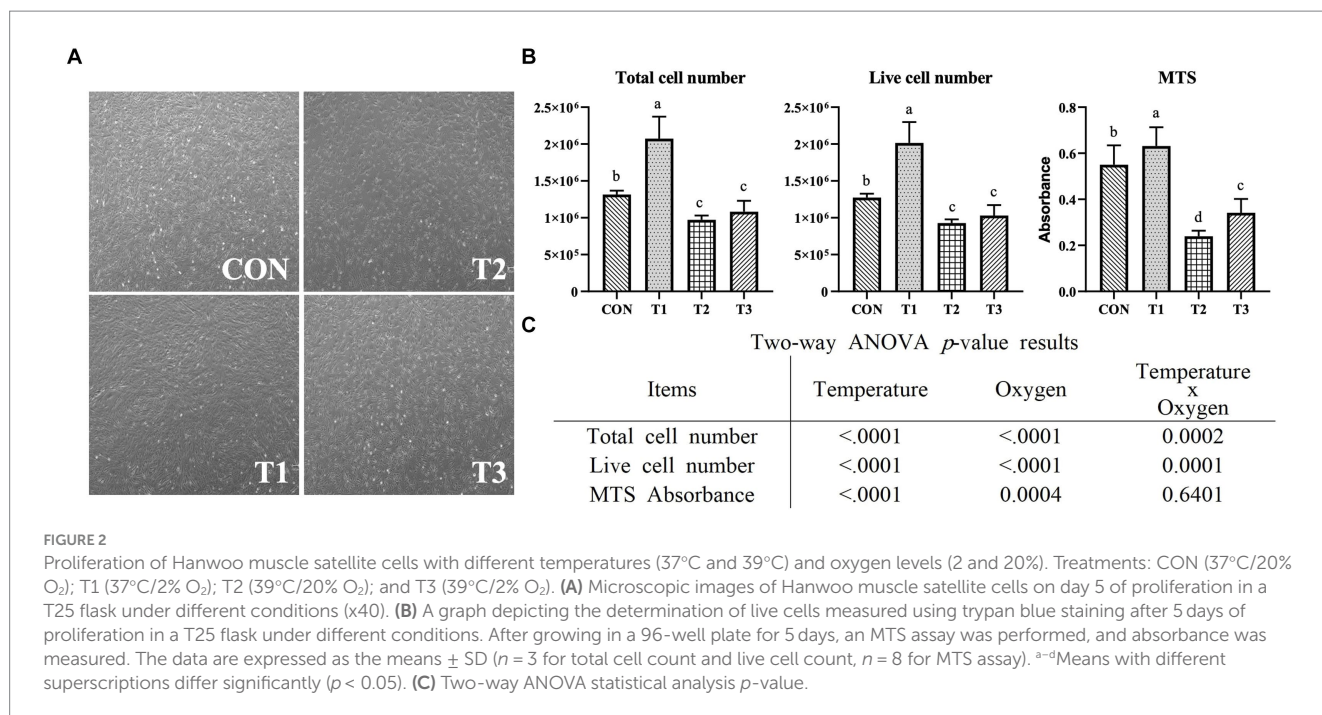
expressions of *MYH2* ( $p < 0.0001$ ), *MYOD1* ( $p < 0.0001$ ), *DES* ( $p < 0.0001$ ), *MYOG* ( $p < 0.0001$ ), and *MB* ( $p < 0.0001$ ).

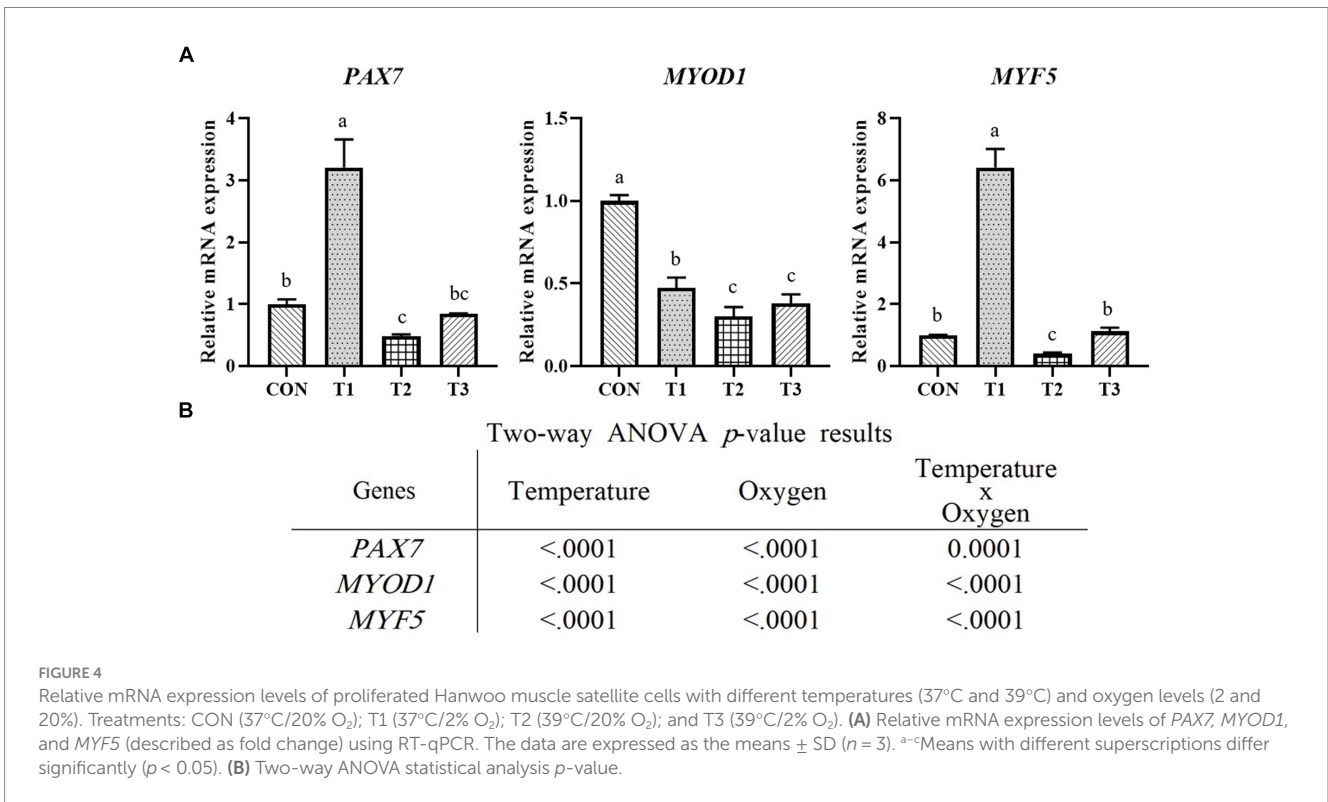
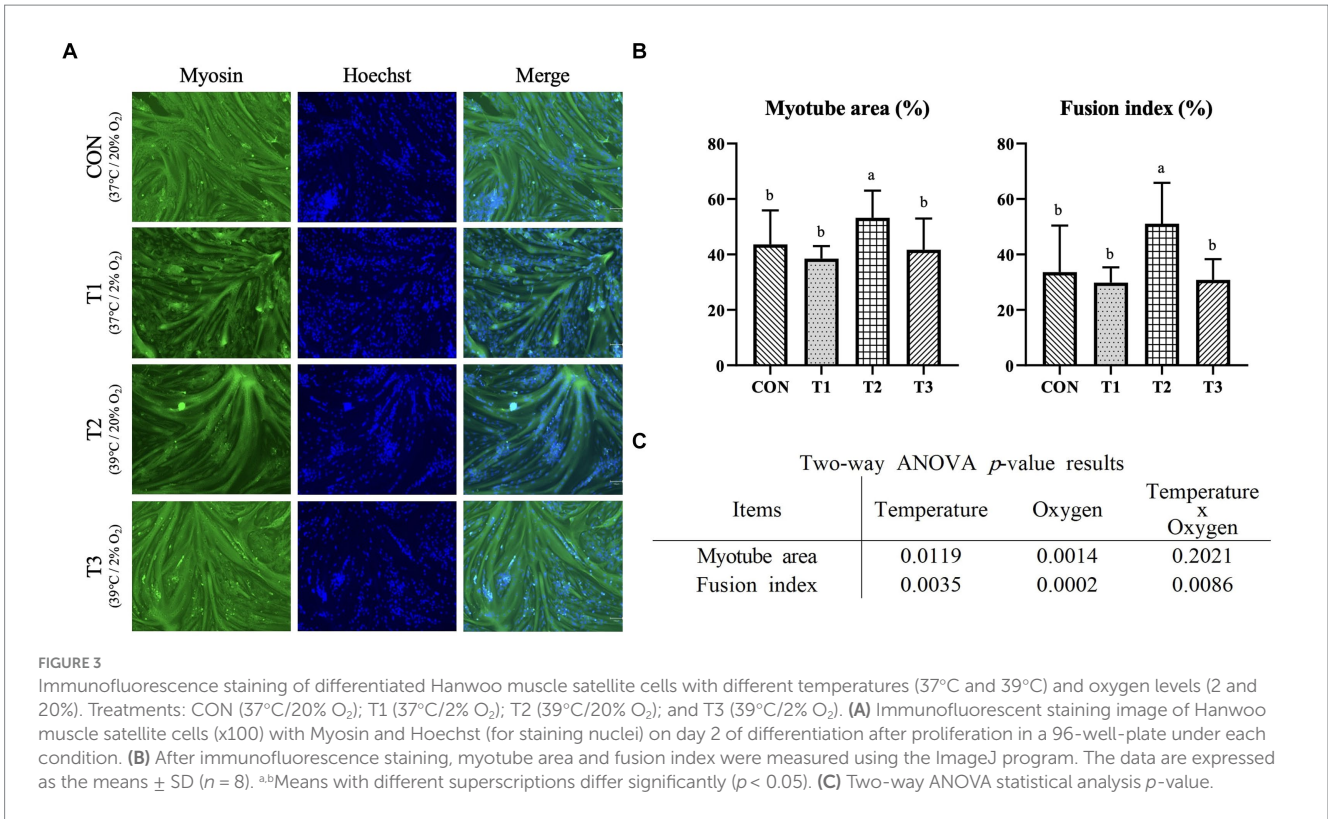
### 3.4 Expression level of muscle-related proteins in the Hanwoo muscle satellite cells

Figure 6A presents the results of fold change for CON by normalizing the fluorescence intensity with that of ACTB, the housekeeping gene. Figure 6B is an image of the fluorescence intensity measured by Western blotting for MYOD1, PAX7, DES, MYOG, and ACTB. The MYOD1 expression exhibited no significant difference ( $p > 0.05$ ). The significantly highest PAX7 expression was observed in T1. Moreover, the treatment group with the same oxygen conditions and a temperature condition of 39°C exhibited significantly lower PAX7 expression than the treatment group with a temperature condition of 37°C ( $p < 0.05$ ). The significantly lowest DES and MYOG expression was observed in T2 ( $p < 0.05$ ), and no significant difference ( $p > 0.05$ ) was observed in CON, T1, and T3. As shown in Figure 6C, temperature and interactive conditions significantly affected the expression of PAX7 ( $p < 0.0001$ ), DES ( $p < 0.0001$ ), and MYOG ( $p < 0.01$ ). The oxygen condition significantly affected the expression of PAX7 ( $p < 0.0001$ ), DES ( $p < 0.0001$ ), and MYOG ( $p < 0.0001$ ).

## 4 Discussion

Our results revealed that temperature and oxygen conditions significantly affect cell numbers. The temperature condition of 39°C decreased the cell number and MTS absorbance of Hanwoo muscle satellite cells compared to the normal temperature and oxygen condition. Furthermore, the RT-qPCR and Western blot results revealed that a culture temperature of 39°C reduces the PAX7 and





MYF5 gene and protein expression in the Hanwoo muscle satellite cells. Conversely, 2% oxygen increased the cell number as well as the expression of PAX7 and MYF5 genes and proteins in these cells. The

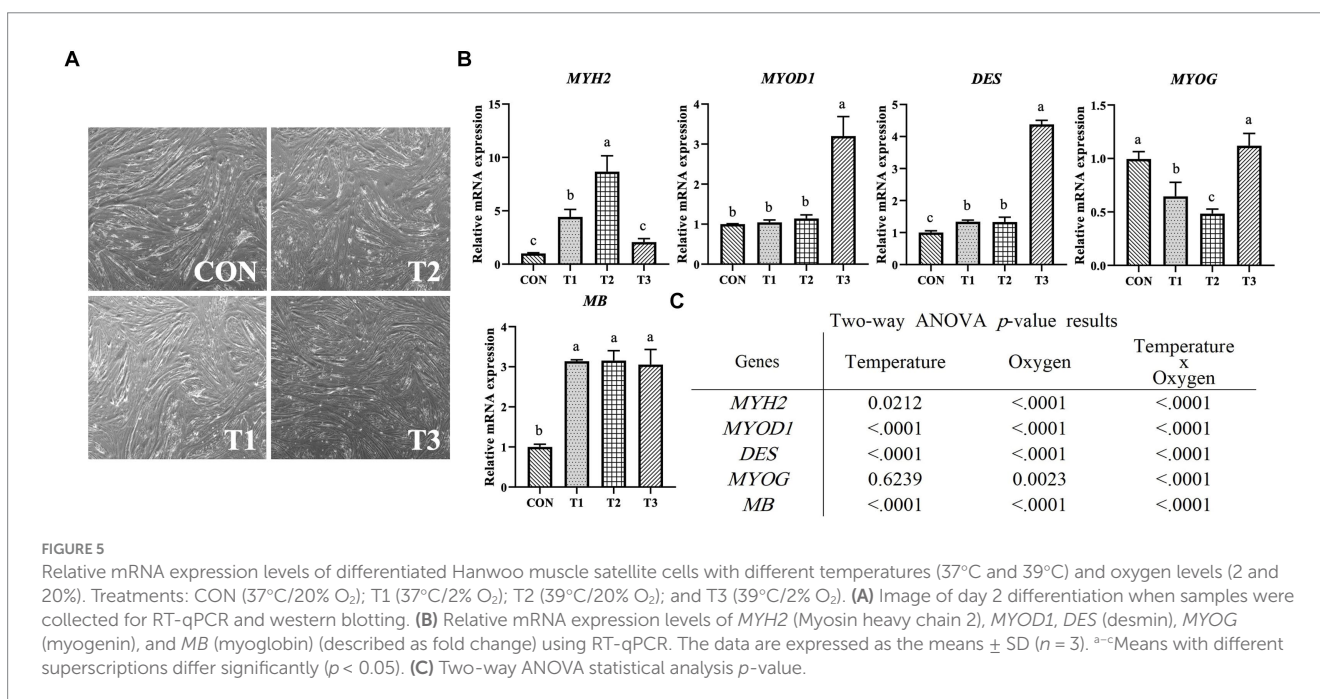
PAX gene family defines a group of transcription factors that play crucial roles during organ organogenesis and tissue homeostasis (Seale et al., 2000; Olguín and Pisconti, 2012; González et al., 2016).

Among them, PAX7 is responsible for developmental and adult regenerative muscle formation (Collins et al., 2009). Additionally, it is regarded as a key regulator of satellite cell growth and proliferation (Sincennes et al., 2016; Yang et al., 2016). The high expression levels of PAX7 factors increase cell proliferation and prevent premature muscle differentiation (Collins et al., 2009; Zhao et al., 2018). MYF5 is the first MRF expressed during myogenesis (Cai et al., 2020). It is expressed in activated satellite cells and proliferating myoblasts before differentiation and is involved in satellite cell activation and proliferation (Cooper et al., 1999; Gayraud-Morel et al., 2007; Averous et al., 2012). Ustanina reported (Ustanina et al., 2007) that proliferation is delayed in MYF5-deficient satellite cells. Consequently, cell proliferation is reduced because of decreased PAX7 and MYF5 gene and protein expression at 39°C. Conversely, 2% oxygen is considered to promote cell proliferation by enhancing the PAX7 and MYF5 gene and protein expression. Kim et al. (2023) reported that heat treatments at 38.5°C and 41°C enhanced cell proliferation and increased the expression of PAX7 and MYF5 in bovine muscle satellite cells. However, in our study, the culture temperature of 39°C decreased the expression of PAX7 and MYF5, resulting in decreased cell proliferation. These results may be due to differences in donor animals and heat treatment times, but further studies are needed. To summarize the above, we considered that a temperature of 39°C inhibited the proliferation of Hanwoo muscle satellite cells by decreasing the expression of PAX7 and MYF5, whereas 2% oxygen promoted the proliferation of these cells by increasing the expression of PAX7 and MYF5. During differentiation, a temperature of 39°C improved the myotube area and fusion index of Hanwoo muscle satellite cells. RT-qPCR and Western blot results revealed that a temperature of 39°C increases the *MYH2* gene and DES and MYOG protein expression in the Hanwoo muscle satellite cells. The interactive condition increased the *MYOD1*, DES, and

*MYOG* gene expression. *MYOD1* determines the myogenic lineage during development and plays an important role in differentiation (Wei and Paterson, 2001; Langley et al., 2002). Cells progress through development by expressing and reducing various factors during proliferation and differentiation. In addition, proliferation and differentiation are completely different processes, and the expression of factors appears very different (Luo et al., 2021). Therefore, it is considered that the expression levels of the *MYOD1* gene differed between proliferation and differentiation in this study. Desmin is a muscle-specific protein and the major subunit of the intermediate filaments of skeletal muscles (Paulin and Li, 2004). Additionally, it is among the myogenic markers with a functional role in muscle formation and differentiation (Li et al., 1994; Beier et al., 2011). The expression of structural proteins, such as desmin, increases when cells fuse with myotubes (Yablonka-Reuveni and Day, 2011). Myogenin is an essential factor in differentiation and is expressed when myoblasts differentiate into myotubes (Mastroiannopoulos et al., 2012). Myogenin-deficient mice die shortly after birth because of skeletal muscle deficiency (Hasty et al., 1993). Myogenin is also used as an indicator of efficient differentiation (Mancini et al., 2011). Therefore, we considered T2 (39°C, 20% O<sub>2</sub>) to have improved myotube formation because DES and MYOG expression levels, myotube area, and fusion index were significantly higher. Based on these results, we concluded that a temperature of 39°C promoted the differentiation of Hanwoo muscle satellite cells by increasing DES and MYOG protein expression.

## 5 Conclusion

The results of this study revealed that a temperature of 39°C decreased both the cell number and the expression of PAX7 and



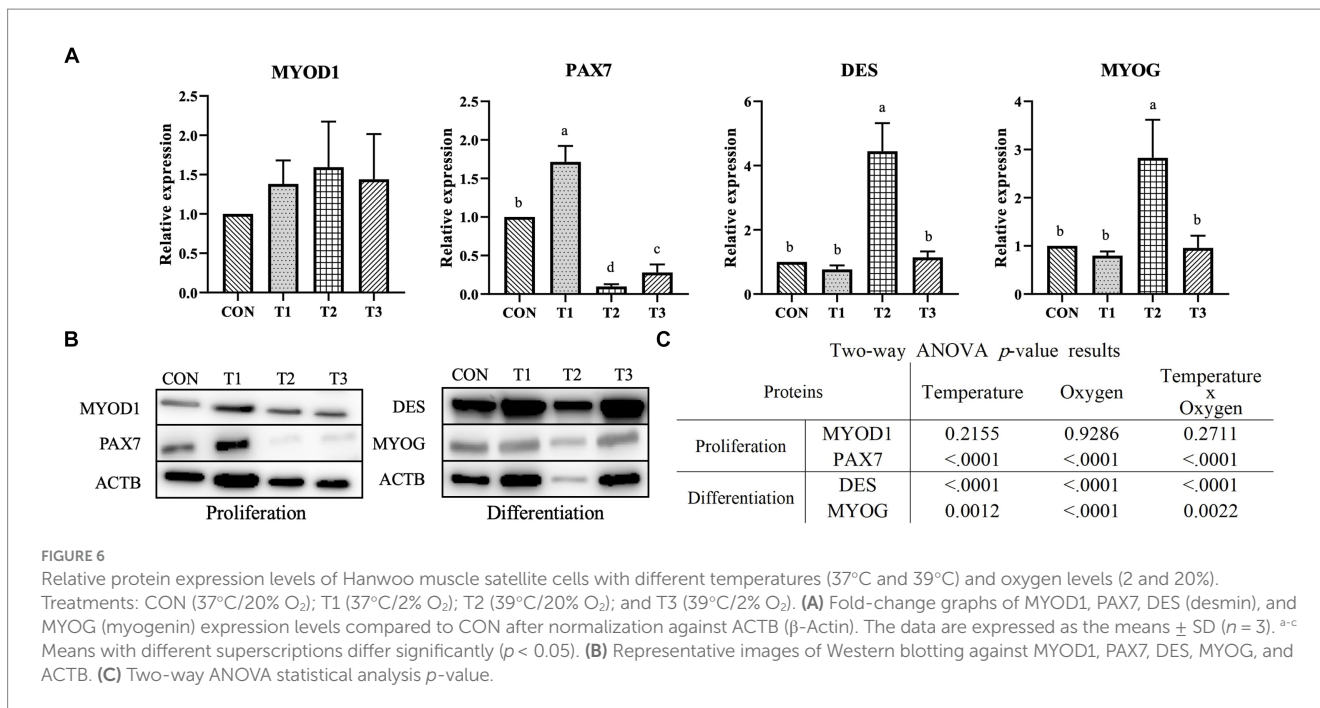


FIGURE 6

Relative protein expression levels of Hanwoo muscle satellite cells with different temperatures (37°C and 39°C) and oxygen levels (2 and 20%). Treatments: CON (37°C/20% O<sub>2</sub>); T1 (37°C/2% O<sub>2</sub>); T2 (39°C/20% O<sub>2</sub>); and T3 (39°C/2% O<sub>2</sub>). (A) Fold-change graphs of MYOD1, PAX7, DES (desmin), and MYOG (myogenin) expression levels compared to CON after normalization against ACTB (β-Actin). The data are expressed as the means ± SD ( $n = 3$ ). <sup>a-c</sup> Means with different superscriptions differ significantly ( $p < 0.05$ ). (B) Representative images of Western blotting against MYOD1, PAX7, DES, MYOG, and ACTB. (C) Two-way ANOVA statistical analysis  $p$ -value.

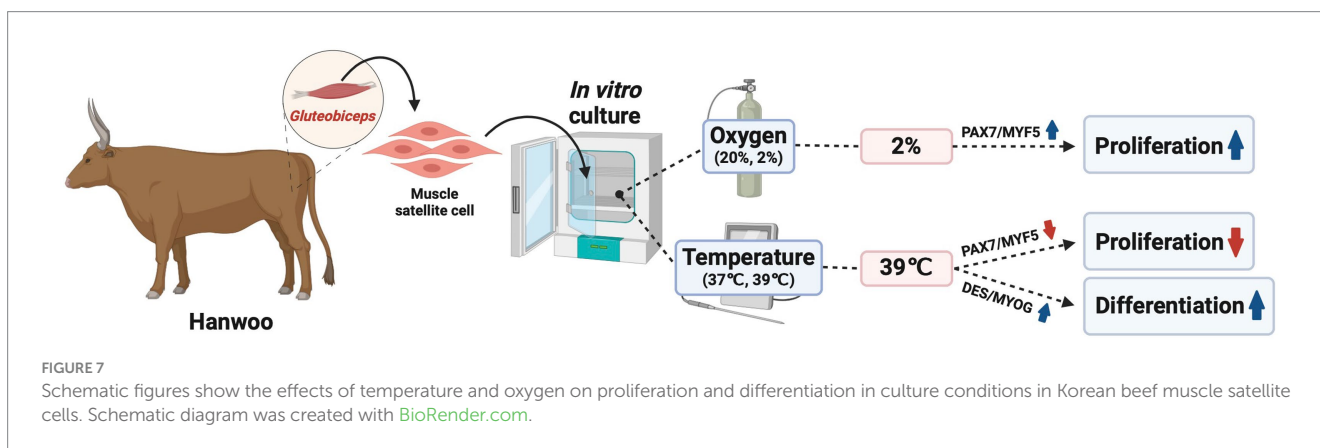


FIGURE 7

Schematic figures show the effects of temperature and oxygen on proliferation and differentiation in culture conditions in Korean beef muscle satellite cells. Schematic diagram was created with BioRender.com.

MYF5. Conversely, 2% oxygen had the opposite effect. Based on the aforementioned results, we concluded that a temperature of 39°C inhibits the proliferation of Hanwoo muscle satellite cells by reducing the expression of PAX7 and MYF5. Conversely, 2% oxygen promoted the proliferation of these cells by enhancing the expression of PAX7 and MYF5. During differentiation, a temperature of 39°C improved the myotube area and fusion index. The RT-qPCR and Western blot experiment results revealed that the culture temperature of 39°C increased the *MYH2* gene and DES and MYOG protein expression. Additionally, the interactive condition increased the *MYOD1*, *DES*, and *MYOG* gene expression. Based on these results, we noted that a temperature of 39°C promoted the differentiation of Hanwoo muscle satellite cells by increasing the DES and MYOG protein expression. Thus, 2% O<sub>2</sub> for proliferation and 39°C for differentiation are expected to be efficient for producing cultivated meat using Hanwoo muscle satellite cells (Figure 7). However, when these conditions

are used for cultivated meat production, additional research is warranted because temperature- and oxygen-related economic aspects must also be considered.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because cells were isolated from meat obtained from a commercial slaughterhouse.



## Author contributions

GP: Data curation, Investigation, Project administration, Resources, Visualization, Writing – original draft. SP: Formal analysis, Methodology, Validation, Writing – review & editing. SO: Data curation, Investigation, Visualization, Writing – review & editing. S-HL: Formal analysis, Methodology, Validation, Writing – review & editing. JC: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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