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Bovine muscle satellite cells in calves and cattle: A comparative study of cellular and genetic characteristics for cultivated meat production

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ABSTRACT

This study compared the cellular and genetic characteristics of bovine skeletal muscle satellite cells (SMSCs) from Hanwoo (a Korean native cattle breed), including calves and mature cattle. SMSCs were isolated using magnetic-activated cell sorting (MACS) from tissue samples of six Hanwoo (three calves and three mature cattle) using the CD29 antibody. Calves' SMSCs exhibited significantly faster growth rates than did those from cattle ($P < 0.01$), with a doubling time of 2.43 days. Genetic analysis revealed higher MyoD and Pax7 expression in SMSCs from calves during proliferation than in those from mature cattle ($P < 0.001$). However, FASN and PLAG1 expression levels were higher in mature cattle than in calves during both proliferation and differentiation ($P < 0.001$). These findings highlight the need for strategies to improve bovine muscle cell growth to produce competitive cultivated meat at a competitive price.

1. Introduction

Meat is a valuable source of essential nutrients such as proteins, fats, and minerals, which are required for basic metabolism (Parlasca and Qaim, 2022). Based on information from the Food and Agriculture Organization (FAO), the global population is projected to reach to around 8.5 billion by 2030, 9.7 billion by 2050, and 10.4 billion by 2100 (FAO, 2022). Furthermore, driven mainly by population and income growth, global meat consumption is expected to increase by 14% by 2030, compared to the consumption levels from 2018 to 2020 (Nation, 2022). Therefore, global meat production is anticipated to rise by approximately 1.2% by 2022, compared to that in 2021 (FAO, 2022). The livestock sector is a major contributor to pressing environmental problems (Petrovic et al., 2015), as it consumes vast amounts of resources to produce meat products such as water, food, electricity, and cleaning chemicals (Petrovic et al., 2015).

To address this issue, various alternative protein sources, such as plant-based meat, edible insects, and cultivated meat, have emerged. These alternatives have the potential to meet the growing demand for meat while also addressing the traditional challenges faced by the livestock industry (Alexander et al., 2017). However, there are challenges with these alternative protein sources. Plant-based meat products currently lack the sensory qualities of traditional meat (H. J. Lee et al., 2020). Similarly, although edible insects are a promising protein source, increased consumer awareness is needed for their widespread consumption (Wilkinson et al., 2018). Cultivated meat is an alternative protein source produced by culturing animal-derived cells *in vitro* and differentiating them into muscles that can be consumed (Alexander et al., 2017). This technology provides consumers with a positive perception of food safety, animal welfare, and livestock diseases (Post, 2014), making it a promising alternative to traditional meat.

Satellite cells, which are myogenic stem cells found in skeletal muscle tissue, are responsible for muscle regeneration, self-renewal, and hypertrophy (E. J. Lee et al., 2007; Stout et al., 2022; Young et al., 2014). The presence of various cell types in muscle tissue, including fibroblasts, blood cells, epithelial cells, and endothelial cells, necessitates the extraction of pure muscle satellite cells for accurate data analysis (Guan et al., 2022). Currently, two methods are used to isolate these cells: fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS). The use of FACS allows for more accurate analysis using flow cytometry, although it can be harsh on the cells during separation. On the other hand, MACS is less harmful during the separation process and is more suitable for large-scale expansion (Choi et al., 2020). To date, several markers, such as cluster of differentiation 29 (CD29; integrin β1), CD34, and CD56 (neural cell adhesion molecule, NCAM), have been used to classify muscle satellite cells (Ding et al., 2017).

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Myogenesis is primarily regulated by four myogenic transcription factors from the myogenic regulatory factor (MRF) family, such as Myf5, Myoblast determination protein 1 (MyoD), MRF4, and Myogenin (MyoG) (B. Kim et al., 2022). During embryonic muscle development, muscle progenitor cells enter the myogenic lineage by expressing MyoD (Wardle, 2019). Paired box gene 7 (Pax7) is also an MRF expressed in satellite cells of postnatal skeletal muscle (Zammit et al., 2006). Pleomorphic adenoma gene 1 (PLAG1) affects animal size and growth. Peripubertal body weight and growth rate have also been shown to be significantly associated with PLAG1 (Littlejohn et al., 2012). Fatty acid synthase (FASN) is associated with beef acid composition which is related to developing beef texture, carcass yield, and beef quality (S.-W. Kim et al., 2010). Myosin heavy chain (MYH) is a motor protein of thick muscle filaments (Wells et al., 1996). The age-dependent difference in cells is related to cell aging speed and cell activation. Parker argued that aged cells are active from the perspective of metabolism but lose the ability to differentiate (Parker, 2015). Furthermore, this could lead to decreased self-renewal ability and the loss of cells or tissues. Thus, the younger the cell, the faster its proliferation and differentiation (Schultz and Lipton, 1982).

Native Korean cattle are recognized for their excellent meat quality and high marbling levels (Cho et al., 2005). In this study, we analyzed the growth, differentiation, and gene expression of myogenic satellite cells from the muscles of Hanwoo cattle and calves. We hypothesized that the age of cattle is closely related to the growth and differentiation abilities of their muscle satellite cells. It is widely accepted in physiology that young cattle have higher growth rates and differentiation abilities in their muscle satellite cells due to increased expression levels of genes such as MyoD and Pax7 that regulate cell growth and development.

2. Materials and methods

2.1. Muscle satellite cell isolation and cell culture

Biceps femoris muscles excised from six Hanwoo cattle (three 2-week-old calves and three 30-month-old cattle) were obtained from Farmstory Hannaeng Bio&Food Co., LTD (Chungbuk, South Korea). Muscle samples were collected and washed with Dulbecco's phosphate-buffered saline (98% DPBS; Welgene, Gyeongsan, South Korea) containing 2% Antibiotic-antimycotic (A.A; Gibco, Gaithersburg, MD, USA) and stored at 4 °C. The muscle samples (30 g per sample) were then rinsed once with 70% ethanol and two to three times with DPBS. After washing with DPBS, the muscle was minced using a meat grinder and 0.8 mg/mL of pronase solution (Sigma P5147, St. Louis, MO, USA) was added to 50 mL conical tubes. The tissue was suspended in the enzyme solution using inversion and the resulting tissue-enzyme suspension was incubated for 40 min at 37 °C in a water bath with the mixture being vortexed every 10 min. After incubation, the tubes were centrifuged at 1200×*g* for 15 min, the pellet was retained, and the supernatant was discarded. Dulbecco's Modified Eagle Medium/Nutrient Mixture (DMEM-F12) (Gibco, Gaithersburg, MD, USA) was added to the tubes and centrifuged at 300×*g* for 5 min. The supernatant was filtered using a 100 μm cell strainer (Corning, New York, NY, USA) and collected in clean 50 mL conical tubes. DMEM-F12 containing 10% fetal bovine serum (FBS; Gibco) was added and centrifuged at 1200×*g* for 15 min. The supernatant was discarded and replaced with stock media (DMEM-F12, A.A, FBS, and dimethyl sulfoxide). The resulting cell preparations were stored in cryovials, allocated so that cells derived from 1 g of muscle was in each vial.

2.2. Magnetic-activated cell sorting (MACS) and immunofluorescence staining

Muscle satellite cells were isolated from whole tissue using a MACS Cell Separation System (Miltenyi Biotec, Bergisch Gladbach, Germany). Once the cells reached 80% confluence, they were collected using 0.05% trypsin-EDTA. The dissociated muscle satellite cells were allowed to react with an anti-CD29 antibody (1:100; eBioscience™, San Diego, CA, USA) and anti-mouse IgG microbeads (1:5; Miltenyi Biotec) and were then sorted using an MS column following the manufacturer's instructions. Sorted CD29-positive cells were counted using a hemocytometer and seeded at a density of 2×10^4 cells/mL in a six-well plate.

For immunofluorescence staining, the cells were preincubated at 4 °C for 10 min and fixed in 4% (w/v) paraformaldehyde for 30 min. After washing twice with DPBS, the cells were treated with 0.2% Triton-X-100 (Sigma-Aldrich) at room temperature for 15 min. The cells were then blocked for 1 h at room temperature with 10% (v/v) donkey serum in DPBS to prevent non-specific binding. Primary antibody CD29 (1:100; 14-0299-82, eBioscience™) was used and incubated overnight at 4 °C. After incubation with the primary antibody, the cells were washed three times with DPBS containing 0.1% Tween 20. The cells were then treated overnight at 4 °C with the appropriate Alexa Flour™ 488 donkey anti-mouse secondary antibody (1:500; R37114, Invitrogen, Waltham, MA, USA). The nuclei were stained with Hoechst 33,342 (Molecular Probes, Eugene, OR, USA). Images of the stained cells were captured using an inverted microscope (CKX53; OLYMPUS, Tokyo, Japan).

2.3. Cell proliferation assay

Calf and cattle muscle cells were seeded at 2 x 10^4 cells/mL in 12-well plates. Muscle cells were cultured in growth medium (GM) consisting of DMEM-F12 containing 1% A.A and 10% FBS. After a 24 h culture period, the GM was replaced with fresh GM, and the cell count was monitored for 1 d. The medium was replaced every 48 h.

Trypsinization for cell counting was performed using 0.05% trypsin-EDTA (Gibco, Gaithersburg, MD, USA) at days 1, 3, 5, and 7 of the experimental period. The cells were counted using a hemocytometer (Counting Chamber; Paul Marienfeld GmbH & Co., Wöllerspfad, Germany). The experiment was performed in triplicate for each sample and the results were averaged each day.

2.4. Cell differentiation assay

Calf and mature cattle muscle cells were seeded at 3 x 10^4 cells/mL in a 12-well plate to assess myogenic cell differentiation. When the cells reached 80–90% confluence, the medium was changed to promote differentiation of the cells. The differentiation media (DM) contained DMEM-F12 with 2% horse serum (Gibco, Gaithersburg, USA), and 1% A.A.

When the cells began to differentiate, their morphology was examined at 0 h. Cell differentiation was assessed after 24, 48, 72, and 96 h. The cells were stained with May-Grünwald solution (Sigma-Aldrich, MO, USA) and Giemsa stain solution (Sigma-Aldrich). After removing the medium, cells were washed twice with DPBS. Then, 100% methanol was added for cell fixation and the cells were allowed to stand for 10 min. Then, methanol was removed and the May-Grünwald solution was added for 5 min. Deionized water (D.W) was added for dilution. After removing the solution, Giemsa staining solution was added (1:20) for 20–30 min. Finally, the solution was removed and the stained cells were observed under a microscope (CKX53, OLYMPUS, Tokyo, Japan).

2.5. Gene expression analysis

The total RNA was extracted from calf and mature cattle muscle cells using Trizol reagent (Accuzol™ Total RNA Extraction Reagent, Bioneer Corporation, Seoul, Korea). For each 10 cm^2 cell culture dish area, 1 mL of Trizol reagent, and 200 μL of chloroform was added. The extraction process was carried out according to the manufacturer's protocol. After extraction, 20 μL of DEPC water (Bioneer Corporation, Seoul, Korea) was added. After checking the purity and concentration of RNA (μDrop™, Thermo Fisher Scientific, USA), cDNA was synthesized using a cDNA reverse transcription kit (AccPower CycleScript RT PreMix, Bioneer

Table 1
The primer sequences used for quantitative real-time PCR.

Gene	Primer	Annealing Temperature	Product size (bp)
GAPDH	F: 5′-ACTCTGGCAAAGTGGATGTTGTC-3′ R: 5′-GCATCACCCCACTTGATGTTG-3′	54	143
PAX7	F: 5′-TGCCCTCAGTGAGTTCGATT-3′ R: 5′-CGGGTTCTGACTCCACATCT-3′	53	180
MYOD	F: 5′-CCGACGGCATGATGGACTA-3′ R: 5′-CTCGCTGTAGTAAGTGCGGT-3′	53	80
PLAG1	F: 5′-GACTCTCCAGCATCCTCGTC-3′ R: 5′-GAAGAGTGTGCCTCCTCCTG-3′	53	236
FASN	F: 5′-ACCTTGACACGGCTCAACTC-3′ R: 5′-CACTGTGGCCATAGGTGGG-3′	54	132
MYOG	F: 5′-AGAAGGTGAATGAAGCCTTCGA-3′ R: 5′-GCAGGCGCTCTATGTACTGGAT-3′	53	114
MHC 1	F: 5′-CCCACTTCTCCCTGATCCACTAC-3′ R: 5′-TTGAGCGGGTCTTTGTTTTTCT-3′	53	82

Corporation) on a GeneAMP PCR System 9700 (Applied Biosystems, Singapore). Quantitative real-time PCR was performed by AccuPower® 2X Greenstar qPCR MasterMix (Bioneer Corporation) and StepOnePlus Real-Time (RT) PCR system (Applied Biosystems). All RT-PCR analyses were performed according to the manufacturer's instructions (Bioneer Corporation). All samples were analyzed in triplicate, and the primer sequences for housekeeping, target genes, and cycling temperatures are listed in Table 1. All data was used to analyze in ΔΔCt methods. For calculating ΔCt values, subtract the Ct value of the housekeeping gene from Ct of the target gene. Next, ΔΔCt values were obtained by subtracting the ΔCt value of the cattle group as control from the ΔCt value of the calf group. Relative quantity was calculated using the formula by $2^{-\Delta\Delta Ct}$ (J. Kim et al., 2018).

2.6. Statistical analysis

All data collection experiments were conducted in triplicate and the results are expressed as the mean ± standard deviation. All data were subjected to two-way Anova using Prism v9.4.0 (GraphPad) and differences were considered significant at a threshold of $P < 0.05$ (*$P <$ 0.05, **$P < 0.01$, and ***$P < 0.001$).

3. Results and discussion

3.1. Identification of specific antibody against muscle satellite cells using immunofluorescence staining

Muscle tissue contains various cell types such as fibroblasts, blood cells, epithelial cells, and endothelial cells (Simsa et al., 2019). Therefore, without purification the ratio of muscle satellite cells decreases, and the amount of myotube fusion is reduced. Ding et al. confirmed the expression of Pax7, an undifferentiated marker of myogenic precursor cells, in CD29^{-}and CD56-positive cells isolated from bovine skeletal

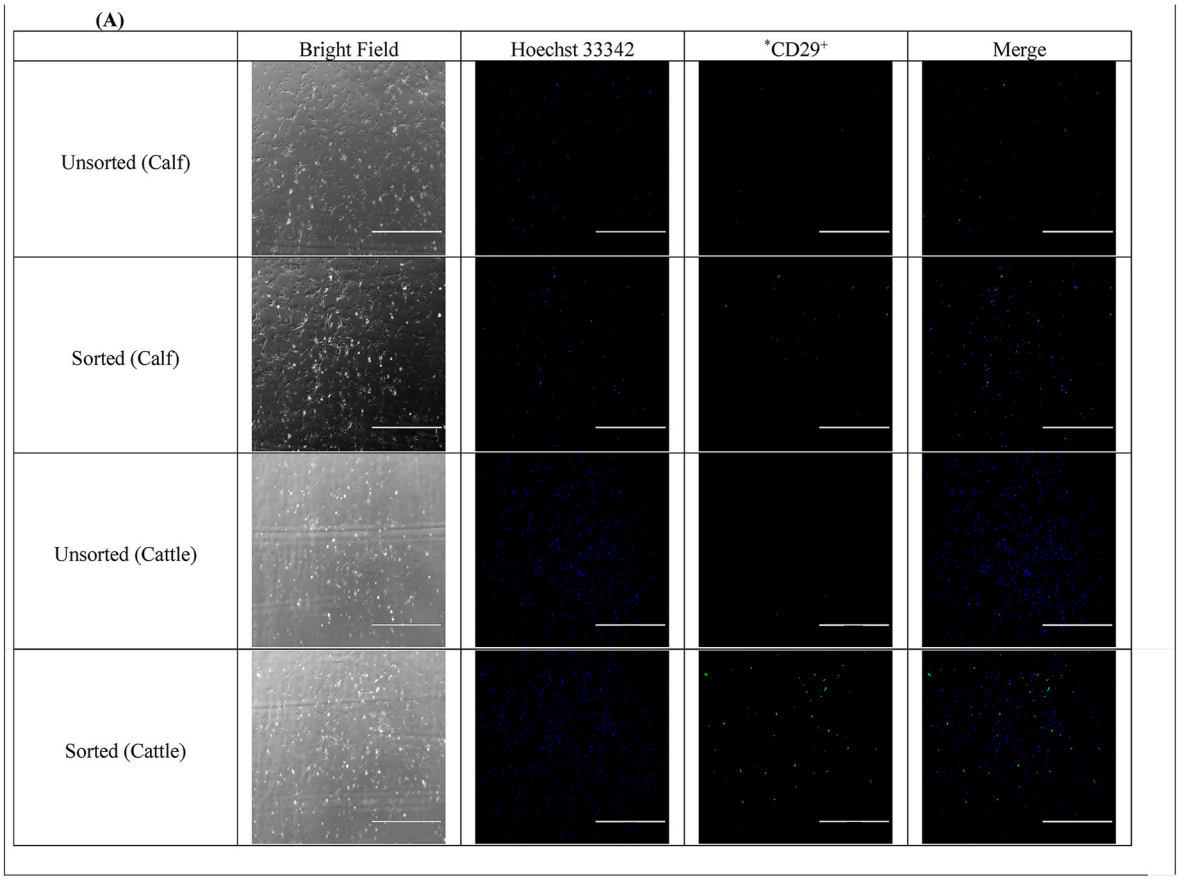

Fig. 1. Purification of bovine muscle satellite cells using the magnetic-activated cell sorting (MACS) with CD29 antibody. (A) Immunostaining was used to visualize the CD29 positive cells among cell populations extracted from calf and cattle. Blue and green fluorescence colors representing nuclei and CD29, respectively. (B) Cells isolated from calf or cattle were counted after sorting. (C) Percentage of nuclei and CD29 positive cells over combined cell population were analyzed. Data are presented as mean ± SD. Statistical difference in cell percentage (%) is indicated as '*' between unsorted and sorted groups (**<0.01, ***<0.001). Scale bar = 1000μm. *CD, Cluster of differentiation.

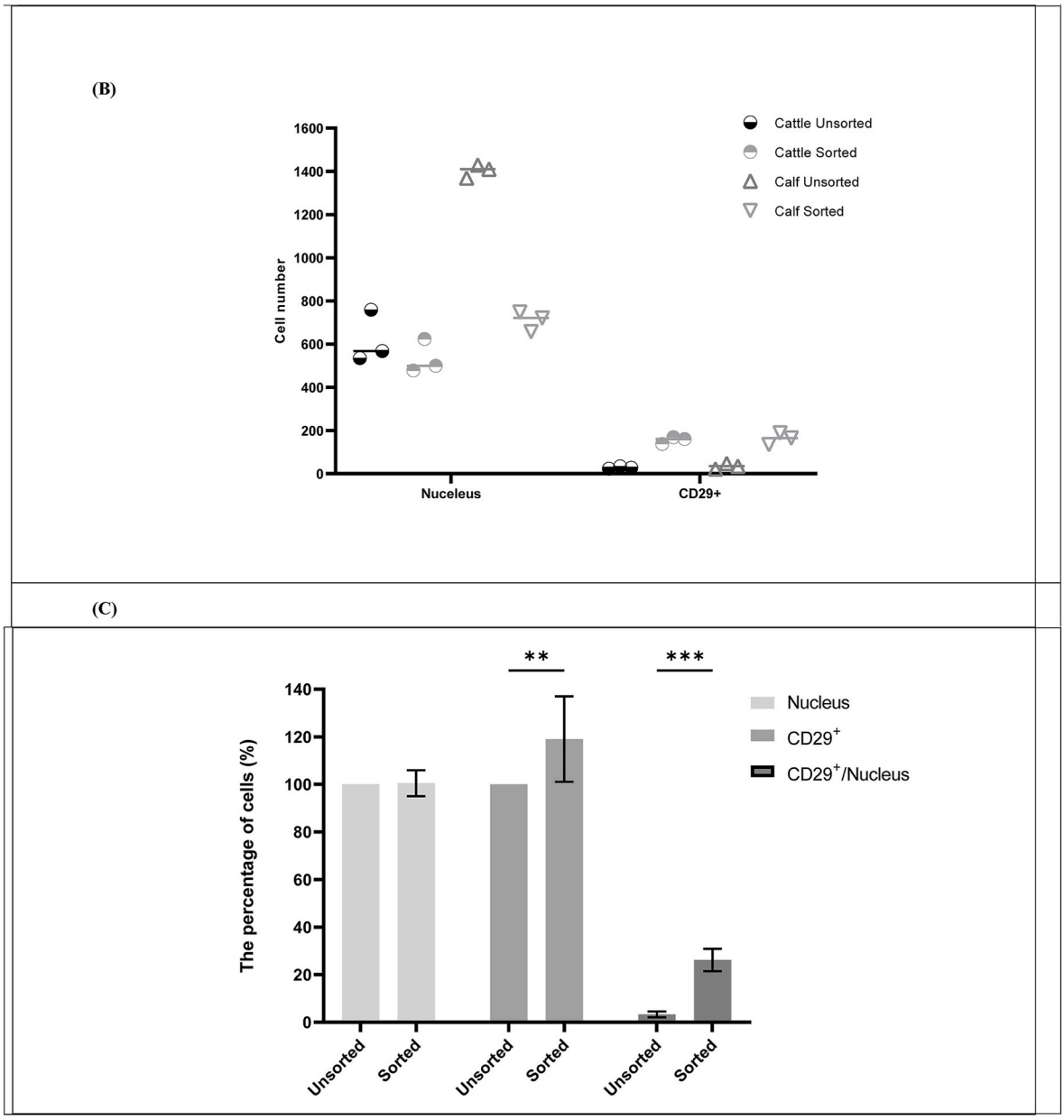

Fig. 1. (*continued*).

muscle tissue using FACS (Ding et al., 2018). The authors also demonstrated that the undifferentiated state of bovine satellite cells could be maintained using a p-38 inhibitor (Ding et al., 2018). MACS has significant advantages in purifying a small number of cells with high efficiency and recovery rates, minimized physical damage to the cells, and increased cell survival rates (Zhu and Murthy, 2013). Although FACS has been extensively studied, there are limited studies on MACS. Choi et al. reported that when using MACS, over 90% of muscle cells isolated from pig tissues were CD29-positive (Choi et al., 2020). Based on this finding, we conducted experiments to determine whether the CD29 antibody was also highly expressed in calves and mature cattle.

Fig. 1A shows the results of the immunofluorescence staining of CD29-positive cells and cell nuclei after MACS, with significant differences observed between the unsorted and sorted groups ($P < 0.05$). Using ImageJ software, the number of cell nuclei was counted (Fig. 1B), revealing the difference in cell nuclei between the unsorted and sorted groups in the calf and mature cattle samples. Counting of CD29-positive cells showed that there were more cells in the sorted group, indicating the presence of many nonmyogenic cells in the unsorted samples. Fig. 1C confirms the significant differences ($P < 0.001$) in the percentage of cells between the unsorted and sorted groups in terms of the nucleus count, CD29-positive count, and CD29-positive/nucleus count. Therefore, the CD29 antibody is a promising marker for the isolation of bovine muscle satellite cells. This approach can be utilized to eliminate cells that have lost their ability to differentiate into muscle cells during cell culture.

3.2. Proliferation rates by cell type and specimen age

In cell biology, cells undergo growth and differentiation, leading to changes in their shape and gene expression over time. Additionally, the rate and pattern of cell growth can be influenced by various factors including age, sex, and breed (Choi et al., 2021). The satellite cell content of muscle tissue decreases significantly with increasing age (Snow, 1977). A rapid decrease in the number of satellite cells occurs during the first months after birth; in humans, the satellite cell content decreases 30-fold between the ages of 0 and 18 months (Verdijk et al., 2014).

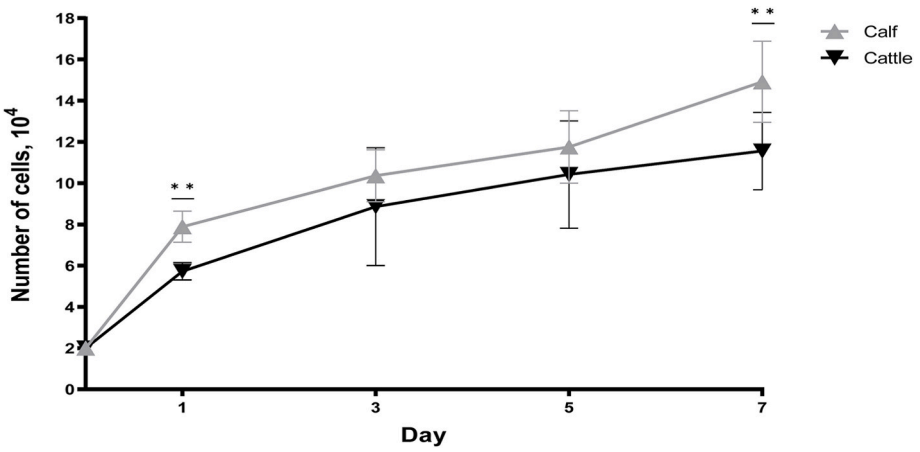

Fig. 2. Changes in cell number between calf and cattle during 7 days-incubation (n = 3 in each group). Results were expressed as Mean ± SD, (**p < 0.01).

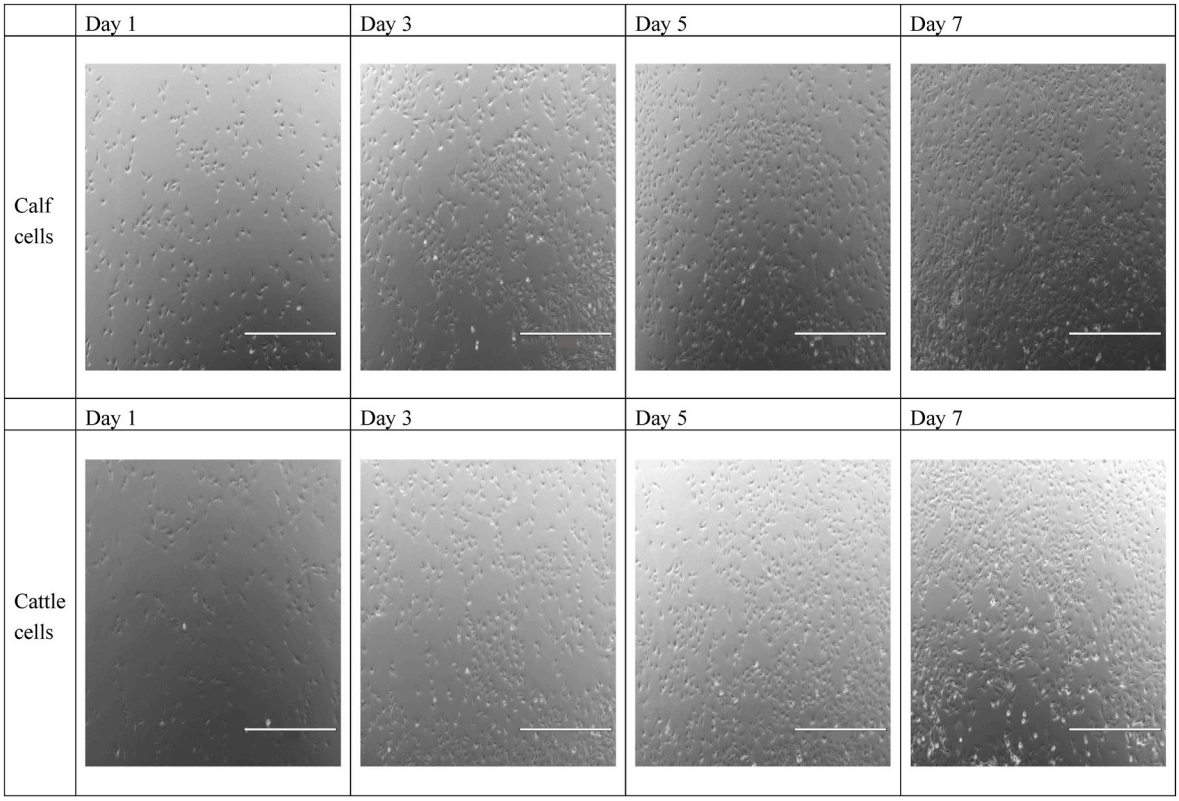

Fig. 3. Representative images of cells isolated from calf and cattle. Scale bar = 1000 μm.

Johnson et al. conducted a comparative analysis of cell growth using C_2C_{12} mouse myoblast cell lines that were cultured for three weeks and nine months, and the results showed that cells from the third week grew faster than did those from the ninth month (Johnson and Allen, 1993).

Following the results of Johnson et al., we compared the proliferation of muscle cells from calves and mature cattle, counted the number of cells, and calculated the Population Doubling Time (PDT). Both the calf and mature cattle cells exhibited steady growth patterns. Calf and mature cattle muscle cells were counted on days one, three, five and seven (Figs. 2 and 3); calf cell readings were 2 x10^4, 7.88 x 10^4, 10.3 x 10^4, 11.8 x 10^4 and 14.9 x 10^4 cells/mL, respectively. Cattle cells readings were 2 x 10^4, 5.72 x 10^4, 8.86 x 10^4, 10.4x10^4, and 11.6 x 10^4 cells/mL, respectively. Thus, calf cells proliferated more than cattle cells. In PDT, calf cells took 2.43 days and mature cattle cells took 2.79 days, and calf cells grew faster than cattle cells ($P < 0.05$). The results showed that calf cells were more appropriate for use in meat cultivation than were cattle cells because calf cells grow faster than cattle cells.

3.3. Differences in myogenic cell differentiation by animal age

In species that are relatively mature at birth, myogenic differentiation results in largely complete muscle fiber differentiation in newborn animals. The potential for muscle growth is highly correlated with the number of muscle fibers present in tissue (Robelin et al., 1993).

Muscle fibers are composed of hundreds to thousands of myofibrils, sarcoplasms, sarcolemmas, and nuclei (Che et al., 2021). Myosin, which is abundant in the skeletal muscles, is responsible for muscle contraction. According to Park et al., the rate of differentiation and expression of muscle regulatory factors can be determined by observing the differentiation rate, morphological changes, and number of differentiated

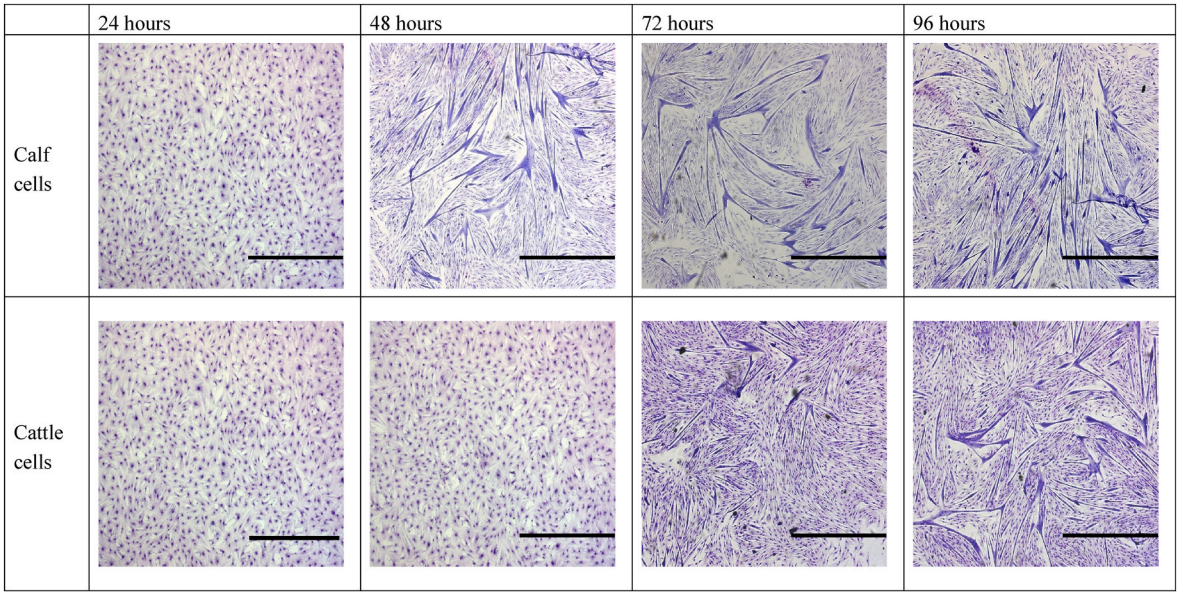

Fig. 4. Morphology of calf and cattle cells stained with Giemsa after 96 h differentiation treatment. Scale bar = 1000 μm.

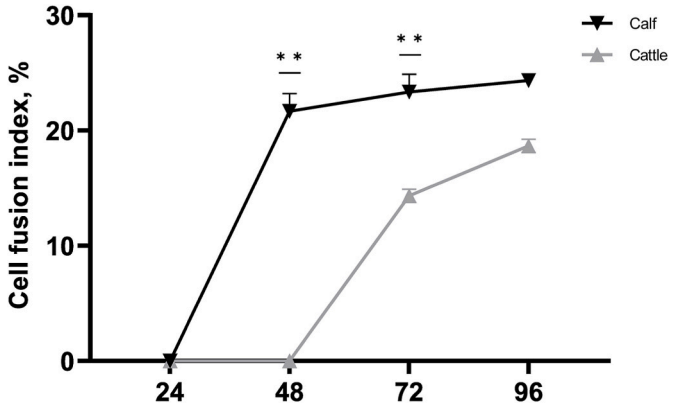

Fig. 5. Cell fusion index of calf and cattle muscle cells after 96 h differentiation. Results were expressed as Mean ± SD, **p < 0.01.

muscle fibers and nuclei (Park and Jeong, 2014).

The differentiated calf and mature cattle cells did not show any myotubes 24 h after changing the DM. In calf cells, myotube formation began after 48 h, and the differentiation rates reached 21.667%, 23.333%, and 24.333% at 48, 72, and 96 h, respectively. On the other hand, in cattle cells, myotubes were not observed until 48 h, but were formed after 72 h, with a differentiation rate of 14.333% at 72 h and 18.667% at 96 h (Figs. 4 and 5). In the calf cells, myotubes did not form until 48 h, but differentiation gradually increased after 72 h (Figs. 4 and 5). The observed differences in the differentiation of calf and mature cattle cells over time can be attributed to the discrepancy in their regenerative capacity, as calf cells possess greater regenerative potential than do cattle cells. This regenerative capability is closely linked to their age difference (Yamakawa et al., 2020).

3.4. mRNA expression profiles across different age groups

Skeletal muscles possess remarkable regenerative abilities and can adapt to physiological demands such as growth or training (von Maltzahn et al., 2014). Under resting conditions, satellite cells are activated, expand, and differentiate during skeletal muscle regeneration, a process controlled by the sequential expression of transcription factors that resembles the differentiation program of embryonic myogenesis. During cell proliferation, Pax7 and MyoD are mainly expressed (Zammit et al., 2006). Pax7 and MyoD belong to the MRF family, which functions as an orchestrating cascade with overlapping actions (Asfour et al., 2018). MyoD, a transcription factor that induces muscle differentiation, is involved in the proliferation and differentiation of muscle satellite cells (Kassar-Duchossoy et al., 2004). The Pax7 gene not only acts as a transcription factor for controlling muscle precursor cells, but is also known to be involved in cell growth (Seale et al., 2000). Once cells enter the differentiation phase, MyoD and Pax 7 gene expression decreased and MYH, which is related to forming myotubes and myofiber, started to be expressed. Among MYHs, MYH1 plays a major role in identifying various muscle fiber types, especially in regulating the size and variation of muscle fibers. In other words, this gene must be highly expressed during muscle differentiation (Henderson et al., 2017). MyoG is responsible for muscle formation and muscle differentiation processes, such as the fusion of myoblasts and activation of muscle genes and differentiation factors (Zammit, 2017). FASN and PLAG1 levels are associated with beef quality and animal size and growth. FASN directly affects the amount of fatty acids, especially oleic acid, an unsaturated fatty acid related to beef quality, flavor, and marbling score (Beak et al., 2019). PLAG1 is a transcription factor that is involved in various cellular during growth and development and is also related to animal weight, growth rate, and meat quality (Littlejohn et al., 2012).

Fig. 6A demonstrates that Pax7 and MyoD expression was greater in calf cells than in mature cattle cells ($P < 0.001$). FASN expression, however, was higher in cattle than calf cells ($p < 0.001$). However, there was no significant difference in PLAG1 expression between calf and cattle cells. Therefore, these results may imply that cells from younger calf can grow faster than mature cattle resulting in producing more cells over time.

As shown in Fig. 6B, all genes showed significant differences between calf and mature cattle cells ($P < 0.001$). FASN and PLAG1 were found to be expressed more in cattle cells than in calf cells ($P < 0.01$)suggesting that mature cattle cells may have advantages over calf cells in terms of beef texture and taste, as their expression pattern is desirable to meat quality.

4. Conclusion

Results from the current study demonstrate that cells from younger calves showed greater capacity of proliferation with increased in related

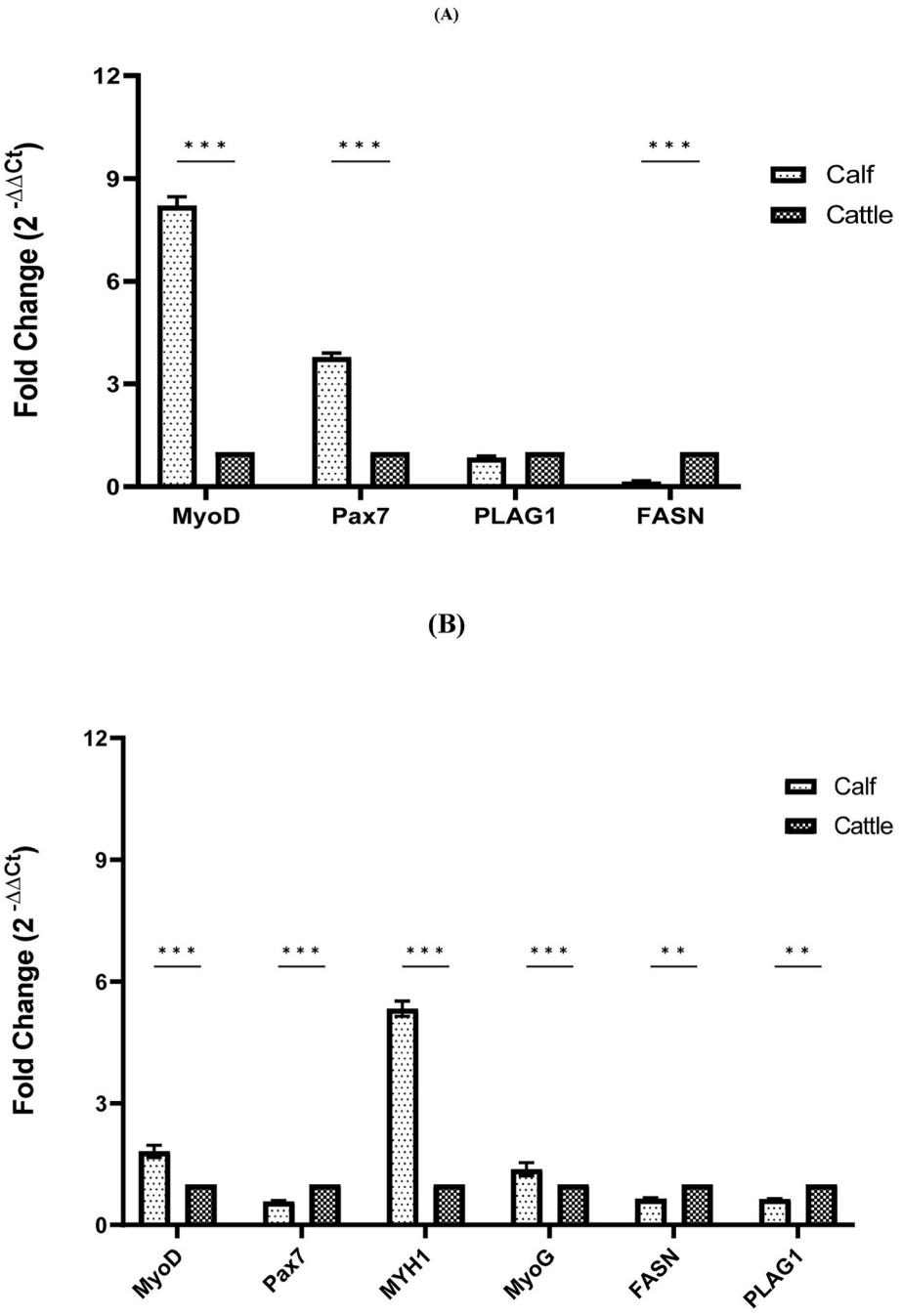

Fig. 6. Relative gene expression of muscle cells isolated from calf and cattle was analyzed by real-time qPCR. (A) Gene expression pattern of calf and cattle muscle cells during proliferation. (B) Gene expression pattern of calf and cattle muscle cells after fully differentiated. Results were shown as mean ± standard deviation (n = 3 in each group, $^{**}p < 0.01$, and $^{***}p < 0.001$).

myogenic gene expression, such as Pax7, MyoD, and MyoG. These results warrant further comparative analysis of the productivity and quality of cultivated meat made of cells derived from skeletal muscles of calf or mature cattle and can hopefully provide useful information for cell-based food production.

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CRediT authorship contribution statement

Bosung Kim: Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Deunsol Ko:** Formal analysis, Methodology, Software, Writing – original draft. **Seong Ho Choi:** Validation, Writing – review & editing. **Sungkwon Park:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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