JAST (Journal of Animal Science and Technology) TITLE PAGE

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Characteristics of Bovine Muscle Satellite Cell from Different Breeds for Efficient Production of Cultured Meat
Running Title (within 10 words)	Characteristics of Bovine Satellite Cell from Different Breeds
Author	Yun-a Kim1#, Sehyuk Oh1#, Gyutae Park1, Sanghun Park1, Yunhwan Park1, Hyunsoo Choi1, Minjung Kim2, Jungseok Choi1 # These authors contributed equally to this work.
Affiliation	1 Department of Animal Science, Chungbuk National University, Cheongju 28644, Korea 2 Food Functionality Research Division, Korea Food Research Institute, Wanju 55365, Korea
ORCID (for more information, please visit https://orcid.org)	Yun-a Kim (https://orcid.org/0000-0002-5505-030X) Sehyuk Oh (https://orcid.org/0000-0003-4105-2512) Gyutae Park (https://orcid.org/0000-0003-1614-1097) Sanghun Park (https://orcid.org/0000-0003-4804-0848) Yunhwan Park (https://orcid.org/0000-0002-2239-6697) Hyunsoo Choi (https://orcid.org/0000-0002-7516-2536) Minjung Kim (https://orcid.org/0000-0003-0205-016X) Jungseok Choi (https://orcid.org/0000-0001-8033-0410)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This work was supported by a grant (715003-07) from the Research Center for Production Management and Technical Development for High Quality Livestock Products through Agriculture, Food and Rural Affairs Convergence Technologies Program for Educating Creative Global Leader, Ministry of Agriculture, Food and Rural Affairs. This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) through High Value-added Food Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (321028-5). This work also was supported by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Education (No. 2020R1A4A1017552, 2020R1G1A1006498).
Acknowledgements	
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Choi JS Data curation: Kim YA, Oh SH, Park GT, Park SH, Park YH Formal analysis: Park SH, Park SH Methodology: Oh SH, Park YH Software: Kim YA, Park GT Validation: Kim YA, Oh SH Investigation: Choi HS, Kim MJ, Writing - original draft: Kim YA, Oh SH Writing - review & editing: Kim YA, Oh SH, Park GT, Park SH, Park YH, Choi HS, Kim MJ, Choi JS
Ethics approval and consent to participate	The animal study protocol was approved by Chungbuk National University Institutional Animal Care and Use Committees (CBNUR-1442-20).

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Jungseok Choi
Email address – this is where your proofs will be sent	jchoi@chungbuk.ac.kr
Secondary Email address	
Address	342 ho, S21-5, 1, Chungdae-ro, Seowon-gu, Cheongju-si, Chungcheongbuk-do, Republic of Korea (28644)
Cell phone number	+82-10-3235-2127
Office phone number	+82-43-261-2551
Fax number	+82-43-273-2240

Abstract

The purpose of this study was comparing in vitro performances of three breeds of donor satellite cells for cultured meat and selecting the optimal donor and providing insight into the selection of donors for cultured meat production. Cattle muscle satellite cells were isolated from the muscle tissue of Hanwoo, Holstein, and Jeju black cattle, and then sorted by FACS. Regarding proliferation of satellite cells, all three breeds showed similar trends. The myogenic potential was higher for Hanwoo and Holstein breeds based on PAX7 and MYOD mRNA expression levels. When the area, width, and fusion index of the myotube were calculated through immunofluorescence staining of myosin, it was confirmed that it was expressed upward in Hanwoo and Holstein. In addition, it was confirmed that Holstein's muscle satellite cells showed an upward expression in the amount of gene and protein expression related to myogenic. In the case of gene expression of MYOG, DES, and MYH4 known to play a key role in differentiation into muscles, it was confirmed that Holstein's muscle satellite cells expressed higher levels. CAV3, IGF1 and TNNT1, which contribute to hypertrophy and differentiation of muscle cells, showed similar trends and showed high expression in Holstein. Our results suggest using cells from Holstein cattle can increase the efficiency of cultured meat production, compared to Hanwoo and Jeju breeds, because the cells exhibit superior differentiation behavior which would lead to greater yields during the maturation phase of bioprocessing.

Keywords:

Cultured meat; Proliferation; Differentiation; Cattle satellite cell

Introduction

The current livestock system has concerns about environmental pollution, sustainability, and animal welfare as a whole [1]. The livestock industry must produce high-quality, low-cost meat in large quantities through environmentally sound, socially responsible and economically viable production systems [2]. The concept of cultured meat is proposed to produce meat without slaughtering livestocks by producing muscle tissues from muscle-derived stem cells in vitro. Cultured meat is the production of meat from animal cells based on the growth and recovery mechanism of animal muscle tissues [1]. After biopsy is taken from living animals, cultured meat can be achieved by separating stem cells from animal muscle biopsy samples and proliferating muscle cells in an environment that provides the energy and nutrients needed for cells to grow inside the animal [1]. Cultured meat has not yet become popular, but cultured meat has several distinct advantages compared to other types of alternative proteins. Cultured meat has the potential to solve these problems. Cultured meat production, once optimized, will produce more meat using fewer resources [3] and could provide cheap sources of rich minerals, vitamins, fats, and amino acids [4]. In Europe, cultured meat was found to have about 82%–96% lower water use, 99% lower land use, and 78%–96% lower greenhouse gas emissions compared to most traditional produced livestock systems [5]. Cultured meat has great potential to eliminate animal suffering and sacrifice during slaughter. In addition, since cells are supplied without slaughter, the pain of slaughter can be eliminated. Cultured meat can be an attractive option for vegetarians, vegans, and opponents who refuse to consume meat for ethical reasons [6]. The number of animals required for cell culture is smaller than that for general meat production [7]. In addition, cultured meat can achieve intensive agricultural reduction without requiring the transport of live animals to slaughter, thus preventing the exponential spread of animal disease [8]. Cultured meat has the potential to significantly reduce animal suffering while meeting all nutritional and hedonic requirements of eating meat [6].

For cultured meat to be used as a reliable alternative to livestock meat, laboratory or factory-grown meat must be produced efficiently. They also must mimic all physical senses such as visual appearance, smell, texture, and, of course, taste of traditional meat [9]. Imitation and efficiency are two key requirements for meat alternatives to be accepted and industrialized [1]. Various types of cells can be used for cultured meat. Pluripotent stem cells and multipotent progenitor cells can also differentiate into muscle cells or fat cells, but they do not differentiate as efficiently as muscle satellite cells and must undergo rigorous safety testing [10]. For this reason, in order to obtain muscle satellite cells repeatedly, muscle tissue must be collected from donor animals.

In the process of producing cultured meat, the choice of animals as stem cell donors has a great influence on efficiency [11]. Since the yield of isolated muscle stem cells depends on conditions of the donor animal, several factors should be considered for more efficient satellite cell separation before selecting the donor animal [1]. The selection of cell donors can have a dramatic impact on the efficiency of the entire process [12]. Thus, it is important to identify the defining characteristics of optimal cell donors. What should be optimized for donor identification includes the following: 1) intensity of proliferation and differentiation; and 2) the lifespan of stem cells. In other words, the ability to continue to multiply while maintaining the ability to differentiate to form mature tissues for meat is important. Donor characteristics for the efficiency of cultured meat should focus on the harvest, efficiency, and quality of muscle satellite cells [12]. Various characteristics of the donor can affect the yield and quality of satellite cells. Melzener et al [13] compared bovine satellite cells isolated from five breeds (Simmental, Limousin, Galloway, Holstein Friesian, and Belgian Blue), and as a result, satellite cells from Limousin and Belgian Blue cattle showed longer retention of differentiation capacity over long term passaging. The proliferation capacity of satellite cells decreases with donor age and varies with donor breeds and disease status [14]. There are major genetic and phenotypic differences between breeds. Certain characteristics, such as maximum size and weight gain rates, are selectively bred to optimize meat and milk production [12]. The carcass properties of cattle are greatly influenced by breeds [15]. Numerous studies have suggested that many aspects of muscle mass and meat quality are closely related to muscle fiber characteristics [16], and there is evidence that differences in muscle fiber characteristics between cattle breeds and cattle are already evident at birth [17]. This can be said that animal breeds affect muscle fiber characteristics, and furthermore, muscle mass and meat quality. In this study, three breeds of cattle mainly used in the Korean livestock industry were compared. A study was conducted to evaluate the suitability of three different breeds of cattle as donor animals for the production of cultured beef based on the proliferation, differentiation, and taste of cell culture.

Materials and Methods

Cattle Muscle Tissue

Satellite cells from Jeju black cattle (*Bos taurus coreanae*) used in the experiment were obtained from semimembranosus muscles of a 41-month-old black cattle at Namwon-eup, Seogwipo-si, Jeju-do, Republic of Korea. Satellite cells from Hanwoo (breed of cattle native to Korea; *Bos Taurus coreanae*) used in the experiment were

obtained from the semimembranosus muscle of a 34-month-old Hanwoo at Farmstory Hannaeng placed in Eumseong-gun, Chungcheongbuk-do, South Korea. Satellite cells of Holstein (*Bos taurus taurus*) used in the experiment were obtained from the semimembranosus muscle of a 16-month-old Holstein at Chungbuk University located in Cheongju-si, Chungcheongbuk-do, South Korea. The animal study protocol was approved by the Institutional Animal Care and Use Committees of Chungbuk National University (CBNUR-1442-20). Tissues were collected from all cattle according to the shipping date.

Isolation of Satellite Cells from Cattle Muscles

Cattle satellite cells were isolated from tissues of each cattle breeds. After washing muscles in alcohol and sterile water, debris attached to these muscles were removed. At 37℃, collagenase type II (600 units/mL) is used to digest cattle muscle fibers in DMEM (11995-065, Gibco, USA) supplement with 3% penicillin-streptomycin-amphotericin B solution antibiotics (PSA, Lonza, Switzerland). To extract cells, muscle satellite cells were separated from muscles and centrifuged. The cells were filtered using a 100 µm cell strainer (431752, Corning® , USA), then a 40 µm cell strainer (431750, Corning® , USA). Until the experiment, harvested muscle satellite cells were kept in liquid nitrogen in cell culture freezing medium (Gibco, Waltham, MA, USA).

Fluorescence Activated Cell Sorting (FACS)

Cells for FACS were grown in a humidified incubator (5% CO2, 37℃) for 5 days on bovine collagen type I (A1064401, Gibco, USA) coated flasks. Cells were suspended in FACS buffer (1:100 bovine serum albumin in phosphate buffered saline) and stained with APC anti-human CD29 antibody (303008, BioLegend, USA), PE-CyTM7 anti-human CD56 (335826, BD, USA), FITC anti-sheep CD31 (MCA1097GA, Bio-Rad, USA), and FITC anti-sheep CD45 (MCA2220GA, Bio-Rad, USA). After antibody incubation, cells were washed multiple times with 1X phosphate buffered saline (PBS) and repaced to Ham's F-10 nutrient mix (11550043, Gibco, USA) with 1% PSA antibiotics and 20% fetal bovine serum, premium (FBS, 2335015CV, Corning, USA). Satellite cells were sorted by filtering for the CD31/CD45-, and CD29+/CD56+ populations.

Culture of Satellite Cells

A flask coated with bovine collagen type I (A1064401, Gibco, USA) for proliferation. Add the collagen coating solution to the flask, and placed in an incubator at 37℃ for a minimum of 16 hours. It was then dried in preparation

for use in the experiment. Muscle satellite cells sorted using FACS were grown on flask coated with collagen in Ham's F-10 medium (11550043, Gibco, USA) containing 20% FBS (16000-044, Gibco, USA), 1% PSA (17-745E, Lonza, USA) and 5 ng/mL basic fibroblast growth factor (bFGF, 13256029, Gibco, USA) as growth medium. Satellite cells were sown at a density of 1,800 cells/cm^2 and grown at 37℃ with 5% CO^2. Cells were passaged every 6 days.

For muscle satellite cell differentiation, flasks were coated with Matrigel (354234, Corning, USA). Cold 1X PBS at a 1:200 ratio was used to make matrigel coating solution. Add the collagen matrigel solution to the flask, and placed in an incubator at 37℃ for a minimum of 4 hours. Following removal of the Matrigel coating solution, washed once with 1X PBS. The medium for cell proliferation was the same as the medium for proliferation mentioned above. Satellite cells were sown at a density of 1,800 cells/cm^2 and changed differentiation medium (DMEM supplemented with 2% FBS and 1% PSA) every 6 days. Cells were grown with 5% CO^2 at 37℃ for 5 days.

Immunofluorescence Staining of Cultured Satellite Cell

In a coated 96-well plate, the satellite cells were seeded at a density of 5,000 cells/cm^2 and cultivated for 5 or 6 days at 37℃ with 5% CO^2. Proliferated or differentiated cells were then washed with 1X PBS after the culture medium was removed. Additionally, cells were exposed to 2% paraformaldehyde (PFA, in PBS) for 45 minutes at 37℃. After washing multiple times with 1X PBS, cells were permeabilized with 0.1% Triton X-100 (in PBS) for 20 minutes at room temperature. Cells were then blocked with 2% BSA for 30 minutes at room temperature, followed by two rounds of washing with 1X PBS. Cell were treated with primary antibody for overnight period at 4℃ in 2% BSA (in 0.1% Triton X-100). The primary antibodies recognizing paired box 7 (PAX7; PA5-68506, Invitrogen, Waltham, MA, USA) and myogenic differentiation (MYOD; bs-2442R, Bioss, USA) and monoclonal anti-myosin (M4276, Sigma, St. Louis, MO, USA). Cells were treated with a secondary antibody at room temperature for 2 hours after being washed multiple times with 1X PBS. Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A21121, Invitrogen) and goat anti-mouse IgG1 cross-adsorbed secondary antibody, Alexa Fluor 488 (A11008, Invitrogen) were used as the secondary antibodies. Finally, Hoechst 33342 reagent (1:2000, H3570, Invitrogen) was added. EVOS-5000 optical microscope was used to examine muscle satellite cells that were Immunofluorescence stained. Using the ImageJ program, skilled experts counted the number of muscle cell nuclei on imaging data.

Reverse Transcription and Quantitative PCR (RT-qPCR)

The satellite cells were sown at a density of 1,800 cells/cm^2 in a coated flask and grown at 37℃ with 5% CO^2 for 5 or 6 days. After removing the cultured medium, proliferated or differentiated cells washed 1X PBS. Muscle satellite cell samples were harvest using cell scrapers. RNA was extracted using the TRIzol reagent. According to the instructions provided by manufacturer, cDNA was created using a Reverse Transcription Master Premix (ELPIS-BIOTECH, Korea) with a template RNA Primer Mixture and 1.0 µg of mRNA as a template, followed by incubation at 60℃ for 60 minutes and 94℃ for 5 minutes. Expression levels of associated genes were examined by quantitative real-time polymerase chain reaction (RT-qPCR). For the examination of expression level, the gene glyceraldehyde-3-phospate dehydrogenase (GAPDH) was employed as an internal control. 1 µl of cDNA, 10 µl EzAmp™ FAST qPCR 2X Master Mix (ELPIS - BIOTECH, Korea), and 1 µl of each primer made up the 20 µl RT-qPCR reaction. After Amplification at 95℃ for 10 minutes, 40 cycles of 95 ℃ for 10 seconds and 61℃ for 20 seconds were perfomed.

Statistical Analysis

All measurements were repeated at least three times. A statistical processing program SAS (9.4 for Windows, USA) was used to test the significance of results. To compare significant differences between measured values, Duncan multiple range test was performed at a significance level of $p < 0.05$.

Results and Discussion

Separation and Expression of in Satellite Cells from Three Breeds of Cattle

To examine the suitability of different cattle breeds as donors for the generation of cultured meat, only satellite cells were extracted. Semimembranosus muscle biopsy samples from each breed have been separated into satellite cells. Satellite cells were extracted using the FACS method. A distinct population of satellite cells (with a CD31-, CD45-, CD29+, and CD56+ immunophenotype) was seen in all three breeds (Fig. 1).

Comparative Analysis of Satellite Cell Proliferation

Proliferation Tendency of Satellite Cells from Three Breeds of Cattle

To determine the proliferation tendency of muscle satellite cells from three breeds of cattle, microscopic photographic analysis was performed (Fig. 2A). After 1,800 cells per cm^2 were seeded in a collagen-coated flask and

cultured at 37℃ for 6 days, satellite cells were subcultured from passage 4 to passage 12. The observed proliferation rate tended to decrease with long-term subculture. It decreased rapidly toward the second half of subculture (passages 7 to 12). However, there was no significant difference in the proliferation tendency between breeds.

Proliferating Activity of Satellite Cells from Three Breeds of Cattle

To determine the proliferation capacity of satellite cells from three breeds of cattle, the number of nuclei was measured on day 6 of passages 4, 8, and 12 by immunofluorescence staining with Hoechst 33342. In the case of passages 4 and 8, it was confirmed that cells were confluent on the flask on day 6 of proliferation (Fig. 3B). The same result was obtained when the nucleus was immunofluorescence stained (Fig. 3A). On the other hand, in the case of passage 12, cells on day 6 of proliferation tended not to be confluent on the flask. These results showed the same tendency as the number of nuclei in a graph (Fig. 3C). Overall, as the subculture progressed, the number of nuclei decreased, with passage 12 showing a rapid decrease in the number of nuclei. In addition, in the case of satellite cells from Jeju black cattle, passage 12 showed a significantly lower number of nuclei compared to those from Hanwoo and Holstein breeds ($p < 0.05$). This indicated that the proliferation capacity of muscle satellite cells from Jeju black cattle decreased significantly as subculture progressed.

Myogenic Potential of Satellite Cells from Three Breeds of Cattle

PAX7 is expressed in a stationary satellite cell state. Activated satellite cells also maintain PAX7 expression. After the cell cycle is stopped, activated muscle satellite cells undergo differentiation or self-regeneration. PAX7 upregulated muscle satellite cells can reacquire a stationary undifferentiated state [18], which means that muscle satellite cells can continue to proliferate through self-proliferation [19]. Immunofluorescence staining and RT-qPCR of the PAX7 factor were performed to confirm the identification and ability of muscle satellite cells to proliferate. Muscle satellite cells from three breeds of cattle were cultured under the same culture conditions. Experiments were conducted for muscle satellite cells on day 6 of proliferation for passages 4, 8, and 12 ($p < 0.05$). It was confirmed that cells proliferated were muscle satellite cells based on PAX7 immunofluorescence staining (Fig 4A-C). Overall, PAX7 expression in muscle satellite cells from three breeds of cattle tended to decrease during subculture (Fig. 4D). It was found that the expression of PAX7 in passage 12 was significantly lower than that in passage 4 or 8. Thus, the ability of muscle satellite cells to proliferate in passage 12 was greatly reduced. It was found that as the subculture progressed, the cell proliferation power decreased, the expression of PAX7 decreased, and the ability of muscle

satellite cells to proliferate was not activated. Comparing PAX7 expression in satellite cells between breeds, Holstein breed showed higher PAX7 expression in passage 4 and passage 8 (Fig. 4D). Hanwoo muscle satellite cells showed significantly lower expression of PAX7 in passage 4 ($p < 0.05$). However, unlike other breeds, it showed no decrease in expression of PAX7 when cultivated up to passage 8. Thus, its proliferation power was maintained. In passage 12, there was no discernible difference in the three types of muscle satellite cells' capacity for proliferation (Fig. 4D). Overall, considering all passages, the PAX7 expression level of Holstein is higher than that of the other two breeds, so it is estimated that the proliferation potential is also high.

MYOD is a transcription factor that plays an essential role in muscle formation, differentiation, and maintenance during muscle development and regeneration [20]. Myoblast activation is something that MYOD is able to cause. MYOD is one of the muscle control factors that is expressed in dividing myoblasts [21]. The absence of MYOD negatively affects muscle regeneration and delays the transition from proliferation to differentiation of satellite cell-derived myocytes [22]. To confirm the potential for muscle satellite cell proliferation and differentiation into myotubes, MYOD factor RT-qPCR and immunofluorescence staining were conducted. Muscle satellite cells from three different breeds were grown in identical conditions. Experiments were conducted with day 6 muscle satellite cells of proliferation at passages 4, 8, and 12 (Fig. 5A-C). Overall, MYOD expression tended to decrease during subculture (Fig. 5D). It was judged that as the subculture progressed, the cell proliferation power decreased and the possibility that muscle satellite cells could differentiate into myotubes decreased as the passage progressed. Comparing the expression amount of MYOD among the three varieties, there was no significant difference in MYOD expression among the three breeds at passage 4 (Fig. 5D). However, passages 8 and 12 showed significantly higher MYOD expression levels in Holstein and Hanwoo muscle satellite cells than Jeju black cattle muscle satellite cells ($p < 0.05$; Fig. 5D). In the case of early passage, all three breeds had similar proliferation and differentiation potential. When the subculture progressed, differentiation capacity of Hanwoo and Holstein muscle satellite cells increased compared to those of Jeju black cattle muscle satellite cells.

Comparative Analysis of Satellite Cell Differentiation

Differentiation Tendency of Satellite Cells from Three Breeds of Cattle

After several proliferation phases, underlying cells begin to differentiate beyond the cell cycle. The next goal is to differentiate them into skeletal muscle cells with the maximum protein production, that is, hypertrophy. A sufficient number of myoblasts obtained through proliferation will return to a stationary satellite cell state, exit the cell cycle,

and develop into myotubes through inter-cellular fusion [23]. After inducing muscle satellite cell differentiation of each breed, the tendency of myotube differentiation of three breeds was compared through microscopic observation for 5 days (Fig. 6A-C). Differentiated muscle satellite cells in passage 4 began to converge on day 1 and completely formed myotubes on day 3 (Fig. 6A). For passage 12 (Fig. 6C), there was little differentiation of myotubes, with the rate of differentiation being significantly slower than those of passage 4 and 8 ($p < 0.05$). It was judged that as the subculture progressed, the differentiation speed and ability of muscle satellite cells from the three breeds of cattle decreased.

The speed and degree of differentiation of muscle satellite cells in three breeds of cattle were examined. In addition, after muscle satellite cells were differentiated at passages 4, 8, and 12, myosin and nuclear immunofluorescence staining were performed to compare degrees of differentiation of the three kinds of differentiated muscle satellite cells (Fig. 7A). Immunofluorescence staining was performed when three breeds of muscle satellite cells were differentiated. At passage 4, Holstein and Hanwoo breeds' satellite cells had high degrees of differentiation than Jeju black cattle's muscle satellite cells based on immunofluorescence image analysis using ImageJ. After calculating widths of myotubes and areas occupied by myotubes, Holstein's muscle satellite cells showed the highest values (Fig. 7B&C). Fusion index values were also significantly higher for Holstein and Hanwoo breeds' satellite cells ($p < 0.05$; Fig. 7D). Thus, Holstein's muscle satellite cells have a higher degree of differentiation than the other two breeds' satellite cells when all cells are differentiated at passage 4. The ratio of the number of nuclei in myocytes with numerous nuclei is represented by the fusion index. The diameter of the myotube is expressed as its breadth. The percentage of an area that myotubes cover is known as the myotube area fraction. After calculating diameter of myotubes and areas occupied by myotubes at passage 8, muscle satellite cells of Hanwoo showed significantly high levels ($p < 0.05$). The fusion index and myotube width at passage 8 were significantly higher for Holstein and Hanwoo muscle satellite cells than those for Jeju black cattle muscle satellite cells ($p < 0.05$). At passage 4, Holstein muscle satellite cells had significantly higher myotube width, myotube area, and fusion index than those for Jeju black cattle muscle satellite cells, and significantly higher myotube width and myotube area than those of Hanwoo muscle satellite cells ($p < 0.05$). In comparison to slowly developing cattle, faster maturing cattle (Angus compared to Holstein) typically have greater muscle fibers of all kinds [24]. Also, Experiments by Hegarty et al. [25] reported similar trend that early maturing pigs have larger muscle fibers. Even in salmon (Salmo salar L.), early maturing population have longer and greater fibers than late maturing population [26].

Differentiation Capacity of Satellite Cells from Three Breeds of Cattle

To analyze levels of cattle muscle cell differentiation marker mRNA of three breeds, subculture was performed and quantitative comparison of muscle cell differentiation was performed. Myogenin (MYOG), desmin (DES), myosin heavy chain 4 (MYH4), caveolin 3 (CAV3), insulin like growth factor 1 (IGF1), and troponin T1 (TNNT1) in cells at the time of maximum differentiation during differentiation were analyzed. MYOG, MYH4, and DES are all markers of myocyte differentiation. MYOG expression is crucial for the differentiation of muscle cells [21]. MYOG are known as transcription factors for inducing fusion [27]. MYOG is abundant in myotube and muscle fibers. It helps to determine and maintain of types of skeletal muscle fibers [28]. In passage 4, Holstein muscle cells showed significantly higher MYOG expression than other breeds of muscle cells ($p < 0.05$). In addition, in passage 8, Holstein showed significantly higher MYOG expression ($p < 0.05$). It was confirmed that Holstein muscle cells had a higher expression of MYOG, a factor that could control differentiation, than the other two breeds' muscle cells (Fig. 8A). DES encodes subunit proteins of intermediate filaments of skeletal muscle tissues, smooth muscle tissues, and myocardial tissues. It is an extremely abundant intermediate filament protein that is unique to muscles [29]. It is connected to muscle formation, proliferation, differentiation, and fusion of muscles. It is also in charge of ensuring that muscle cells operate normally [30]. During Myogenesis, DES is expressed in differentiated muscle cells, muscle tubes, and muscle fibers. It is one of the crucial indicator of muscle differentiation [31]. In passage4, Holstein muscle cells showed significantly higher DES mRNA expression than the other two breeds' muscle cells ($p < 0.05$). In addition, at passage 8, there was no apparent difference in DES mRNA expression among all breeds of cattle muscle cells (Fig. 8B). The main source of fiber motor protein needed for muscle contraction is MYH protein. The MYH gene produces its encoding [32]. Cells of the skeletal muscle express MYH. Myotube formation can be aided by it. Its expression and more muscle mass are positively associated [33]. Muscle-specific proteins such as DES and MYH4 are important for the contraction function of muscle fibers. MYH4 is a marker that affects muscle mass. It is involved in fusion into myotubes. MYH4 showed significantly higher mRNA expression at passage 4 in Holstein muscle cells than in the other two breeds' muscle cells ($p < 0.05$). In addition, in passage 8, Holstein muscle cells showed significantly higher expression of MYH4 mRNA ($p < 0.05$). On the other hand, Jeju black cattle muscle cells showed low expression of MYH4 mRNA (Fig. 8C). Skeletal muscles are where CAV3 is expressed most frequently. CAV3 contributes to proper differentiation of myofibrils and homeostasis of myofibrils [34]. CAV3 is also expressed in mature multinucleated myotubes. It is mainly expressed in the final differentiation stage. It plays an essential role in the fusion of mononucleated myoblast cells into multi-nucleated myotubes [35]. CAV3 contributes to differentiation and myofibrils homeostasis. It is a

marker of mature multinuclear myotube. CAV3 showed significantly higher expression at passage 4 in Holstein muscle cells than in the other two breeds' muscle cells ($p < 0.05$). Its expression was significantly lower in Hanwoo muscle cells. At passage 8, Holstein muscle cells showed significantly higher expression of CAV3 than the other two breeds' muscle cells ($p < 0.05$). Overall, Holstein's expression was measured to be significantly higher ($p < 0.05$; Fig. 8D). IGF1 can stimulate MRFs such as MYOD and MYOG [36]. IGF1 is allocated to biological processes such as myoplasia, myofascial proliferation, myofascial differentiation, and myofascial fusion [30]. IGF1 also contributes to the development and maintenance of muscular tissue [37]. IGF1 is temporarily expressed after muscle cell differentiation. It can promote muscle fiber enlargement in a self-secreting manner [38]. Higher IGF1 expression levels may contribute to higher muscle mass growth. IGF1 is a marker that stimulates MRF during differentiation. It is involved in muscle hypertrophy. However, at passage 8, Holstein's muscle cells showed significantly higher expression of IGF1 than the other two types of muscle cells ($p < 0.05$; Fig. 8E). TNNT1 is a gene involved in muscle formation, such as MYOG and CAV3. TNNT1, a skeletal muscle marker, is only observed in differentiated cells. TNNT1 is a subunit of tropomyosin binding. It can combine with tropomyosin to form a troponin-tropomyosin complex, which is crucial for controlling the contraction of striated muscle. TNNT1 is a differentiation-specific marker observed in differentiated myotube. At passage 4, TNNT1 showed significantly higher expression in Jeju black cattle muscle cells than in Holstein and Hanwoo breeds' muscle cells ($p < 0.05$). Passage 8 showed the same trend. Jeju black cattle muscle cells showed lower TNNT1 expression at passages 4 and 8 (Fig. 8F). These results can be associated with the characteristics of cell donor breeds.

Among Holstein, Hanwoo, and Jeju black cattle, Holstein breeds show the largest and highest growth. Satellite cells are deeply related to muscle formation and growth in vivo. It is judged that Holstein muscle satellite cells have better proliferation and differentiation capacity in vitro than muscle satellite cells of Hanwoo and Jeju black cattle due to the genetic factors of the breed.

Conclusion

The purpose of this study was to investigate and compare proliferation and differentiation characteristics of satellite cells from three breeds of cattle distributed in Korea. In this study, an experiment was conducted with an emphasis on cattle breeds. However, gender, environmental factors, and conductor age of functional elements are also factors affecting the selection of donors for culture meat production. Although more researchs are needed, this study

emphasized the importance of selecting donors. It sought to identify characteristics of donors for differences between breeds. In this case, Holstein can be optimally selected as satellite cell donors for beef culture meat production due to the faster proliferation potential and stronger differentiation capacity of satellite cells than Hanwoo and Jeju black cattle. It will help us select the best donor to produce beef culture meat in Korea. Results of this study provide insight into the choice of donor for cultured meat.

Acknowledgments

This work was supported by a grant (715003-07) from the Research Center for Production Management and Technical Development for High Quality Livestock Products through Agriculture, Food and Rural Affairs Convergence Technologies Program for Educating Creative Global Leader, Ministry of Agriculture, Food and Rural Affairs. This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) through High Value-added Food Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (321028-5). This work also was supported by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Education (No. 2020R1A4A1017552, 2020R1G1A1006498).

References

1. Post MJ. Cultured meat from stem cells: Challenges and prospects. Meat Sci. 2012;92(3):297-301.

2. Scollan ND, Greenwood PL, Newbold CJ, Ruiz DRY, Shingfield KJ, Wallace RJ, et al. Future research priorities for animal production in a changing world. Anim Prod Sci. 2011;51(1):1-5.

3. Rubio NR, Xiang N, Kaplan DL. Plant-based and cell-based approaches to meat production. Nat Commun. 2020;11(1).

4. Post MJ, Levenberg S, Kaplan DL, Genovese N, Fu JA, Bryant CJ, et al. Scientific, sustainability and regulatory challenges of cultured meat. Nat Food. 2020;1(7):403-15.

5. Mattick CS, Landis AE, Allenby BR, Genovese NJ. Anticipatory Life Cycle Analysis of In Vitro Biomass Cultivation for Cultured Meat Production in the United States. Environ Sci Technol. 2015;49(19):11941-9.

6. Hopkins PD, Dacey A. Vegetarian meat: Could technology save animals and satisfy meat eaters? Journal of Agricultural and Environmental Ethics. 2008;21:579-96.

7. Edelman PD, McFarland DC, Mironov VA, Matheny JG. In vitro-cultured meat production. Tissue Eng. 2005;11(5-6):659-62.

8. Levitt T. Two billion and rising: the global trade in live animals in eight charts. The Guardian. 2020;20.

9. Bredahl L, Grunert KG, Fertin C. Relating consumer perceptions of pork quality to physical product characteristics. Food Qual Prefer. 1998;9(4):273-81.

10. Shaikh S, Lee E, Ahmad K, Ahmad SS, Chun H, Lim J, et al. Cell Types Used for Cultured Meat Production and the Importance of Myokines. Foods. 2021;10(10).

11. Chriki S, Ellies-Oury M-P, Hocquette J-F. Is “cultured meat” a viable alternative to slaughtering animals and a good comprise between animal welfare and human expectations? Animal Frontiers. 2022;12(1):35-42.

12. Melzener L, Verzijden KE, Buijs AJ, Post MJ, Flack JE. Cultured beef: from small biopsy to substantial quantity. J Sci Food Agr. 2021;101(1):7-14.

13. Melzener L, Ding S, Hueber R, Messmer T, Zhou G, Post MJ, et al. Comparative analysis of cattle breeds as satellite cells donors for cultured beef. bioRxiv. 2022:2022.01. 14.476358.

14. Hawke TJ, Garry DJ. Myogenic satellite cells: physiology to molecular biology. J Appl Physiol. 2001;91(2):534-51.

15. Jurie C, Martin JF, Listrat A, Jailler R, Culioli J, Picard B. Effects of age and breed of beef bulls on growth parameters, carcass and muscle characteristics. Anim Sci. 2005;80:257-63.

16. Joo ST, Kim GD, Hwang YH, Ryu YC. Control of fresh meat quality through manipulation of muscle fiber characteristics. Meat Sci. 2013;95(4):828-36.

17. Wegner J, Albrecht E, Fiedler I, Teuscher F, Papstein HJ, Ender K. Growth-and breed-related changes of muscle fiber characteristics in cattle. J Anim Sci. 2000;78(6):1485-96.

18. Olguin HC, Olwin BB. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev Biol. 2004;275(2):375-88.

19. Zammit PS, Relaix F, Nagata Y, Ruiz AP, Collins CA, Partridge TA, et al. Pax7 and myogenic progression in skeletal muscle satellite cells. J Cell Sci. 2006;119(Pt 9):1824-32.

20. Tapscott SJ. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. Development. 2005;132(12):2685-95.

21. Weintraub H. The Myod Family and Myogenesis - Redundancy, Networks, and Thresholds. Cell. 1993;75(7):1241-4.

22. Sabourin LA, Girgis-Gabardo A, Seale P, Asakura A, Rudnicki MA. Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. J Cell Biol. 1999;144(4):631-43.

23. Zhou SP, Han LF, Wu ZG. A Long Journey before Cycling: Regulation of Quiescence Exit in Adult Muscle Satellite Cells. Int J Mol Sci. 2022;23(3).

24. Cornforth DP, Hecker AL, Cramer DA, Spindler AA, Mathias MM. Maturity and Its Relationship to Muscle Characteristics of Cattle. J Anim Sci. 1980;50(1):75-80.

25. Hegarty PVJ, Gundlach LC, Allen CE. Comparative Growth of Porcine Skeletal-Muscles Using an Indirect Prediction of Muscle-Fiber Number. Growth. 1973;37(4):333-44.

26. Johnston IA, Alderson R, Sandham C, Mitchell D, Selkirk C, Dingwall A, et al. Patterns of muscle growth in early and late maturing populations of Atlantic salmon (Salmo salar L.). Aquaculture. 2000;189(3-4):307-33.

27. Dumont NA, Bentzinger CF, Sincennes MC, Rudnicki MA. Satellite Cells and Skeletal Muscle Regeneration. Compr Physiol. 2015;5(3):1027-59.

28. Hughes SM, Taylor JM, Tapscott SJ, Gurley CM, Carter WJ, Peterson CA. Selective accumulation of MyoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. Development. 1993;118(4):1137-47.

29. Hnia K, Ramspacher C, Vermot J, Laporte J. Desmin in muscle and associated diseases: beyond the structural function. Cell Tissue Res. 2015;360(3):591-608.

30. Ciecierska A, Motyl T, Sadkowski T. Transcriptomic Profile of Primary Culture of Skeletal Muscle Cells Isolated from Semitendinosus Muscle of Beef and Dairy Bulls. Int J Mol Sci. 2020;21(13).

31. Kelc R, Trapecar M, Gradisnik L, Rupnik MS, Vogrin M. Platelet-rich plasma, especially when combined with a TGF-β inhibitor promotes proliferation, viability and myogenic differentiation of myoblasts in vitro. PLoS One. 2015;10(2):e0117302.

32. Weiss A, Leinwand LA. The mammalian myosin heavy chain gene family. Annu Rev Cell Dev Bi. 1996;12:417-39.

33. Xu Y, Shi T, Cai HF, Zhou Y, Lan XY, Zhang CL, et al. Associations of MYH3 gene copy number variations with transcriptional expression and growth traits in Chinese cattle. Gene. 2014;535(2):106-11.

34. Hnasko R, Lisanti MP. The biology of caveolae: lessons from caveolin knockout mice and implications for human disease. Mol Interv. 2003;3(8):445-64.

35. Quach NL, Biressi S, Reichardt LF, Keller C, Rando TA. Focal adhesion kinase signaling regulates the expression of caveolin 3 and beta1 integrin, genes essential for normal myoblast fusion. Mol Biol Cell. 2009;20(14):3422-35.

36. Kumar A, Yamauchi J, Girgenrath T, Girgenrath M. Muscle-specific expression of insulin-like growth factor 1 improves outcome in Lama2 Dy-w mice, a model for congenital muscular dystrophy type 1A. Human molecular genetics. 2011;20(12):2333-43.

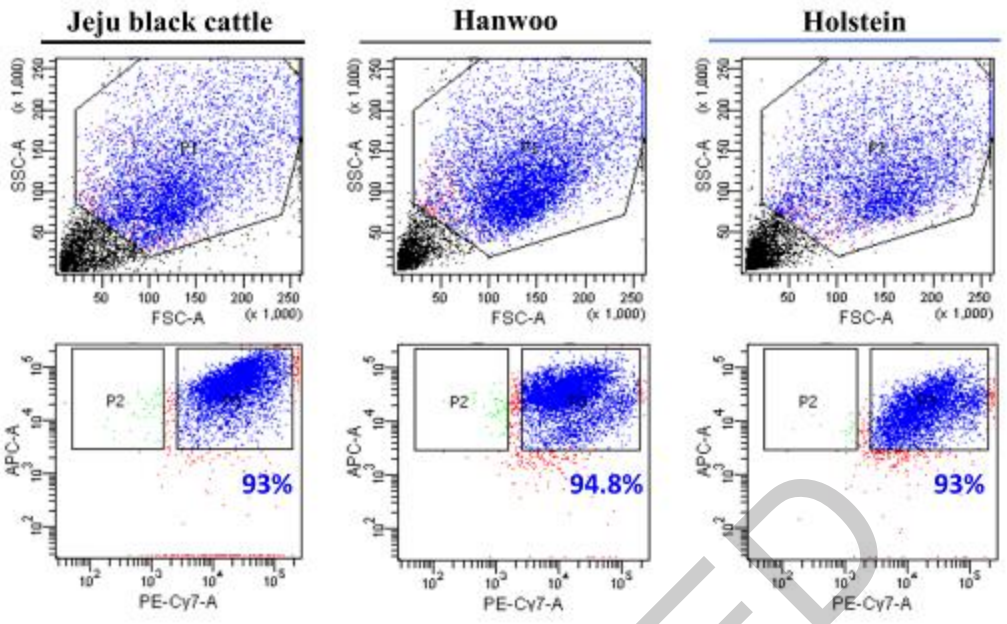
37. Ascenzi F, Barberi L, Dobrowolny G, Bacurau AVN, Nicoletti C, Rizzuto E, et al. Effects of IGF-1 isoforms on muscle growth and sarcopenia. Aging Cell. 2019;18(3).

38. Tollefsen SE, Lajara R, Mccusker RH, Clemmons DR, Rotwein P. Insulin-Like Growth-Factors (Igf) in Muscle Development - Expression of Igf-I, the Igf-I Receptor, and an Igf Binding-Protein during Myoblast Differentiation. J Biol Chem. 1989;264(23):13810-7.

Tables and Figures

Table 1. Sequences of the primers used in the RT-qPCR assays

Primer	Direction	Sequence(5'→3')
MYOD	F	CCCAAAGATTGCGCTTAAGTG
	R	AGTTCCTTCGCCTCTCCTACCT
PAX7	F	CTCCCTCTGAAGCGTAAGCA
	R	GGGTAGTGGGTCCTCTCGAA
MYOG	F	GCGCAGACTCAAGAAGGTGA
	R	TGCAGGCGCTCTATGTACTG
DES	F	GCTGAAAGAAGAAGCGGAGAAC
	R	GAGCTAGAGTGGCTGCATCCA
MYH4	F	AGAGCAGCAAGTGGATGACCTTGA
	R	TGGACTCTTGGGCCAACTTGAGAT
CAV3	F	CCCAGATCGTCAAGGACATT
	R	TGTAGCTCACCTTCCACACG
IGF1	F	TTGGTGGATGCTCTCCAGTTC
	R	AGCAGCACTCATCCACGATTC
TNNT1	F	AGAAGTTCCGGAAGGGGG
	R	ACACGCCAAGGACTCCCA
GAPDH	F	CACCCTCAAGATTGTCAGC
	R	TAAGTCCCTCCACGATGC

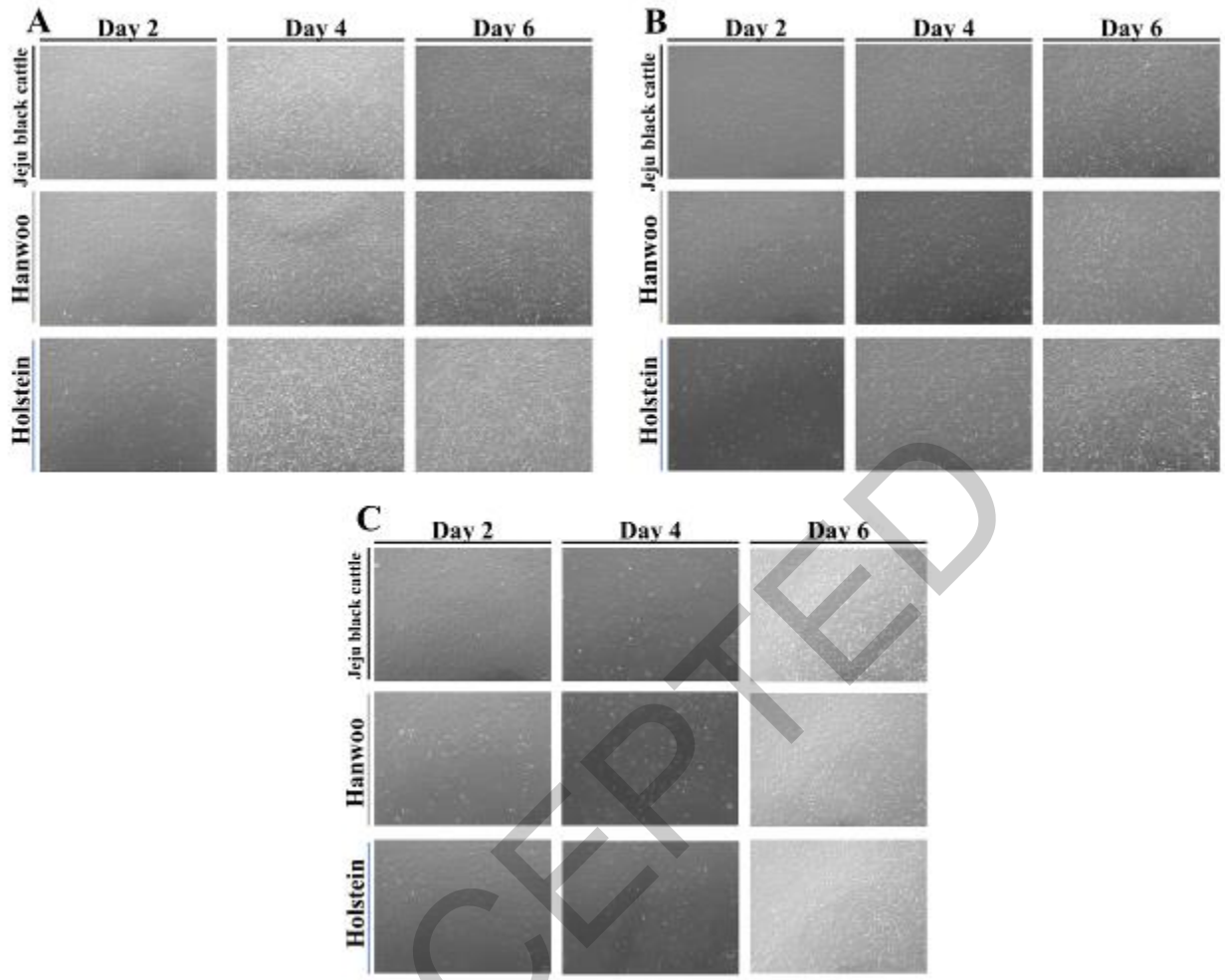


421

422 Figure 1. All cattle breed donors can produce satellite cells that are pure. Using forward/side scatter (FSC/SSC
423 respectively) and surface expression of CD31/CD45 (FITC), CD29 (APC), and CD56 (PE/Cy7), representative flow
424 cytometry plots of unsorted muscle cells from the three breeds of cattle are shown in (A). Fluorescence-activated
425 cell sorting (FACS) is indicated by coloured gates.

426

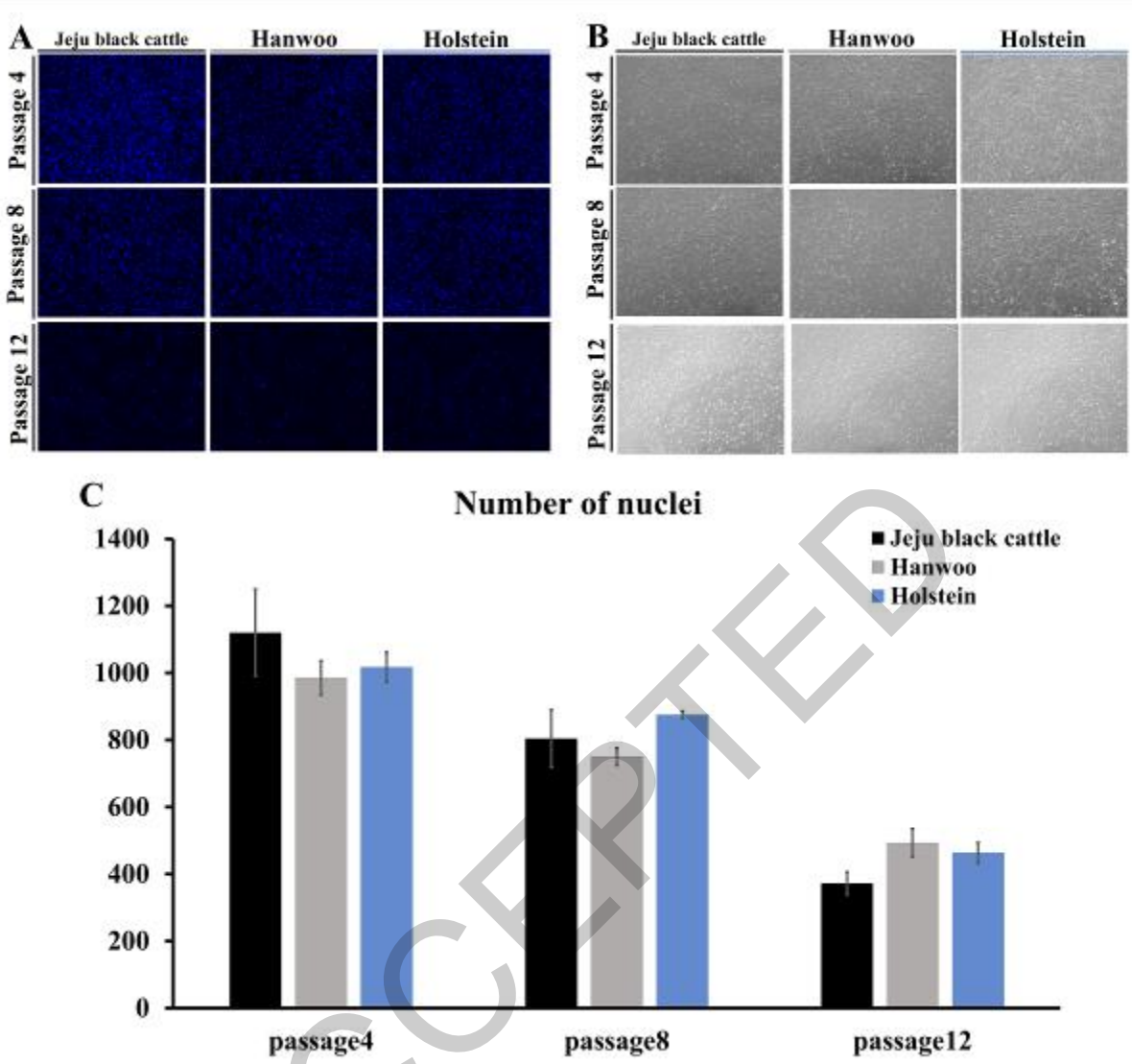
427



428

429 Figure 2. Proliferation of Satellite Cells from Three Breeds of Cattle during Subculture. (A) Representative
430 brightfield microscopy images of satellite cell morphology on day 2, day 4, and day 6 for passage 4. (B) passage 8
431 (C) passage 12 (All images taken at 40×)

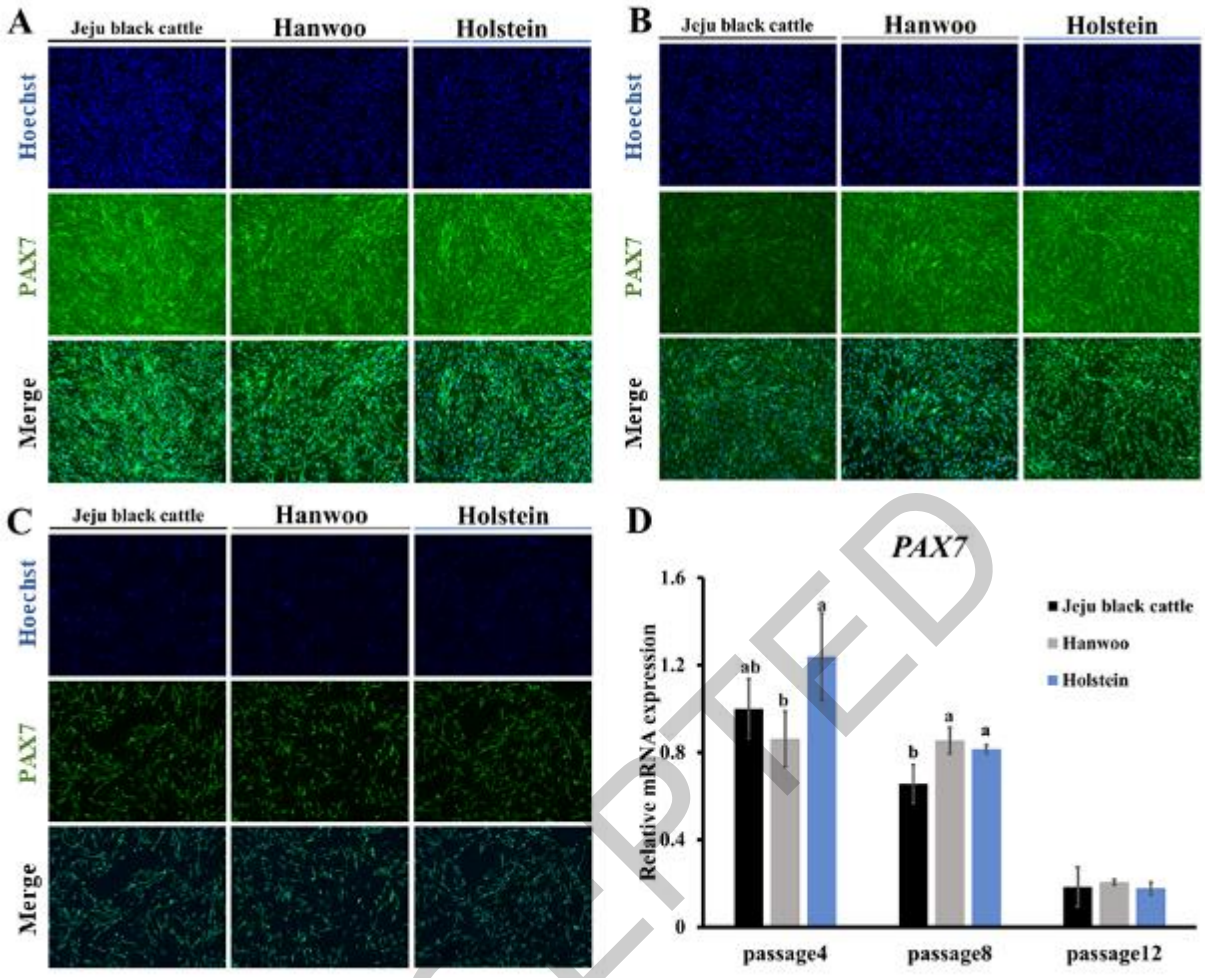
432



433

434 Figure 3. Proliferation Capacity of Satellite Cells from Three Breeds of Cattle. (A) Representative
435 immunofluorescence microscopy image of satellite cells from three breeds of cattle on day 6 for Passages 4, 8, and
436 12 of cell proliferation. (B) Brightfield microscopy images on day 6 for Passages 4, 8, and 12 of cell proliferation
437 (All images taken at 40×). (C) A graph showing the number of nuclei by fluorescent staining on day 6 for Passages
438 4, 8, and 12 of cell proliferation of satellite cells from three breeds cattle. Data are expressed as mean ± SEM (n =
439 9).

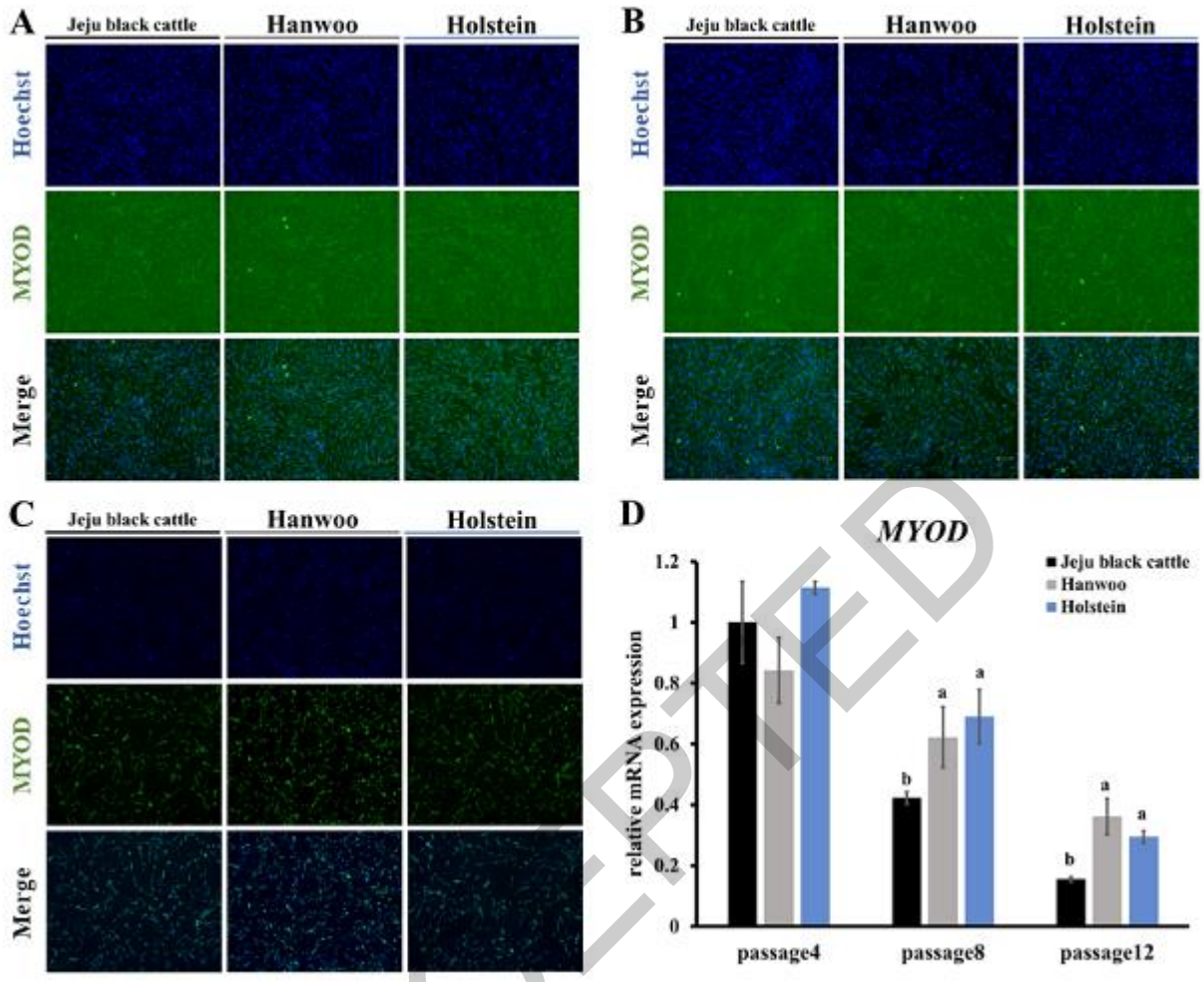
440



441

442 Figure 4. Expression of Nuclei and Satellite Cell Marker PAX7 in Satellite Cells from Three Breeds of Cattle. (A)
443 Representative immunofluorescence microscopy image of the nuclei and PAX7 of satellite cells from three cattle
444 breeds after confluent proliferation at Passage 4, (B) Passage 8, (C) Passage 12 (All images taken at 40×). (D)
445 Expression of *PAX7* genes as measured by RT-qPCR at passages 4, 8, and 12 for each breed. Gene expression was
446 normalized against that in Jeju black cattle on Passage 4. Data are expressed as mean ± SEM (n = 3) with differing
447 letters differ significantly ($p < 0.05$).

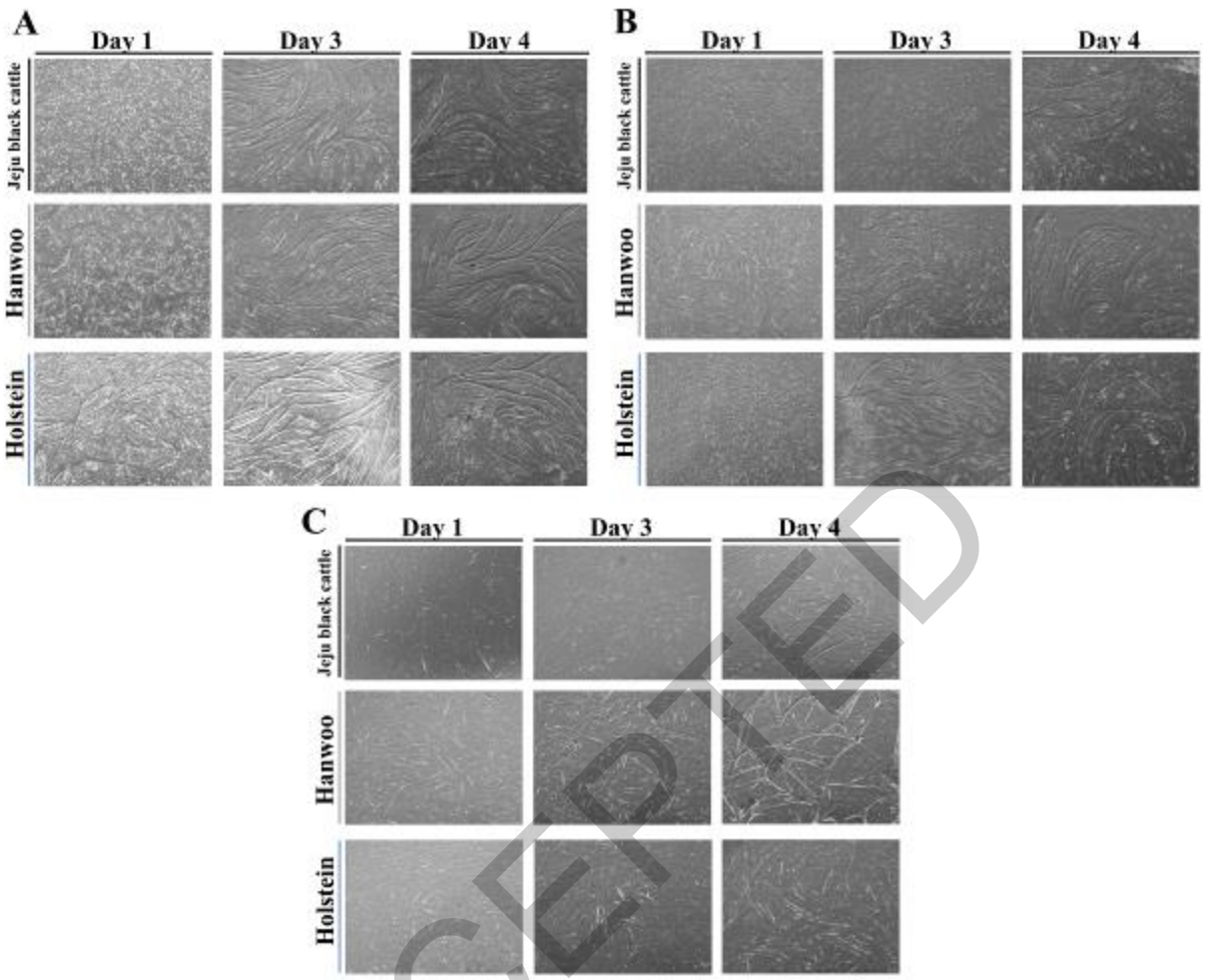
448



449

450 Figure 5. Expression of Nuclei and Satellite Cell Marker PAX7 in Satellite Cells from Three Breeds of Cattle. (A)
451 Representative immunofluorescence microscopy image of the nuclei and PAX7 of satellite cells from three cattle
452 breeds after confluent proliferation at Passage 4, (B) Passage 8. (C) Passage 12 (All images taken at 40×). (D)
453 Expression of *MYOD* genes as measured by RT-qPCR at passages 4, 8, and 12 for each breed. Gene expression was
454 normalized against that in Jeju black cattle on Passage 4. Data are expressed as mean ± SEM (n = 3) with differing
455 letters differ significantly ($p < 0.05$).

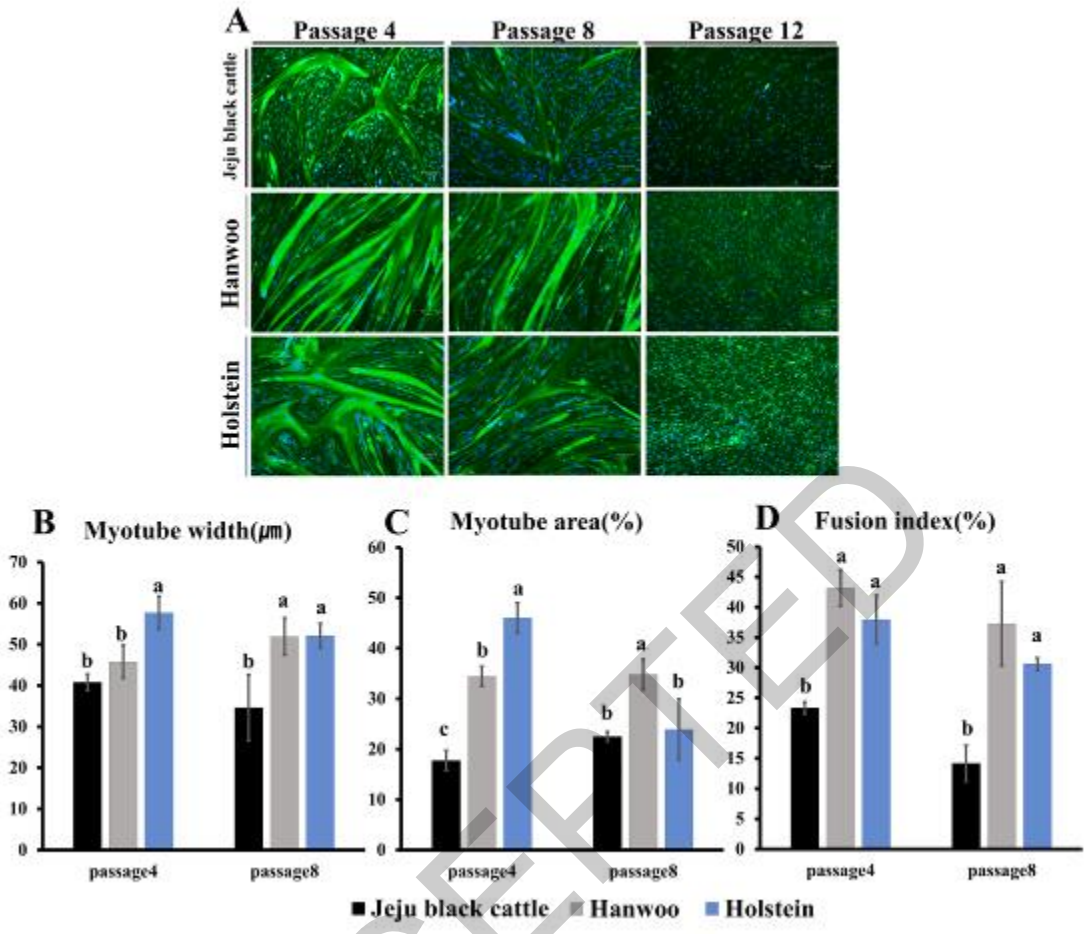
456



457

458 Figure 6. Differentiation of Satellite Cells from Three Breeds of Cattle during Subculture. (A) Representative
459 brightfield microscopy images of days 1, 3, and 4 of myogenic differentiation at Passage 4, (B) Passage 8, (C)
460 Passage 12 (All images taken at 40×)

461



462

463 Figure 7. Differentiation Speed and Degree of Satellite Cells from Three Breeds of Cattle. (A) Representative
464 immunofluorescence microscopy images of satellite cells from three breeds of cattle at passages 4, 8, and 12. Green
465 = myosin, blue = Hoechst. Myotube width, myotube area, and fusion index were determined with ImageJ. For
466 differentiation analysis, only myosin-positive cells with three or more nuclei were rated as myotubes (All images
467 taken at 40×). (B) By converting the number of myotubes with three or more nuclei to a percentage of all nuclei,
468 fusion indices were computed. (C) Myotube areas were measured using ImageJ program. (D) Myotube widths were
469 measured using ImageJ program. Data are expressed as mean ± SEM (n = 9) with differing letters differ
470 significantly ($p < 0.05$)

471

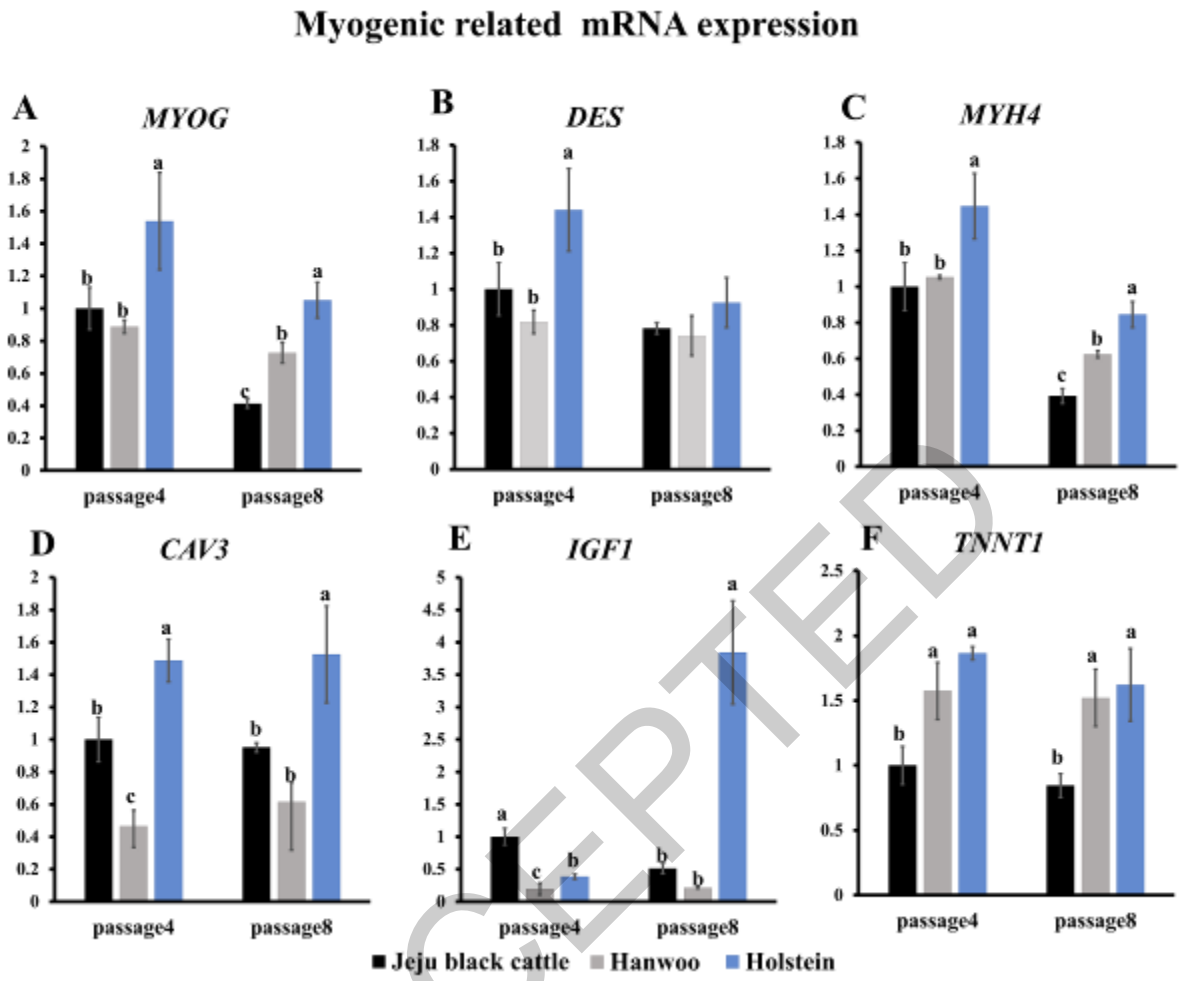


Figure 8. myogenic and Muscle Specific mRNA Expression Profile of Satellite Cell from Three Breeds of Cattle. the expression levels of genes specific for myogenic differentiation were determined by using GAPDH as a housekeeping gene at passages 4 and 8 for each breed. (A) Relative mRNA levels of *MYOG*. (B) *DES*. (C) *MYH4*. (D) *CAV3*. (E) *IGF1*. (F) *TNNT1*. Data are expressed as mean ± SEM (n = 3) with differing letters differ significantly ($p < 0.05$).