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Comparative Analysis of Skeletal Muscle Satellite Cells from Hanwoo Steers and Cows for Optimizing Cell-Based Meat Production

Abstract:

This study aimed to compare the proliferation, differentiation rates, and related gene expression levels of skeletal muscle satellite cells (SMSCs) isolated from Hanwoo steers and cows to identify a suitable source for cell-based meat production. SMSCs were extracted from three steers and three cows, and their cell growth rates, doubling times, differentiation rates, and genetic differences were determined. Comparative analysis revealed that SMSCs from steers exhibited a higher growth rate than cows. Doubling time was shorter in steers than in cows ($p<0.0001$). During differentiation, the cell fusion index showed significant differences between steers and cows ($p<0.0001$). Steers showed differentiation after 24 h, while cows showed differentiation after 72h.Genetic analysis showed that the expression level of the Pax7 gene was significantly higher in steers than in cows ($p<0.001$). The expression levels of differentiation-related genes, including MyoG, MRF4, and MHC1 were higher in steers than in cows. Thus, SMSCs isolated from steer muscle exhibit a faster growth rate, and these findings are expected to provide valuable information for determining the optimal cells for cell-based food.

Keywords: Steers; Cows; Muscle satellite cell; Cell culture; Genetic Analysis

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Introduction

Meat, which constitutes approximately one fourth of the protein intake in the human diet and provides 15% of the energy consumed, plays a vital role in the growth and maintenance of the human body (Alexander et al., 2017). Furthermore, owing to the continuous increase in the global population, meat consumption is steadily increasing, with an estimated 2.55 million tons expected by 2050 (OECD/FAO, 2021). This escalating demand for meat production and consumption raises concerns about environmental issues and resource depletion, highlighting the need for sustainable meat production methods (Rempe, 2022). Issues related to the environment, animal welfare, and health have led to a rapid expansion of research on meat analogue (Green et al., 2022). Among these meat analogue, cell-based foods, edible insects, and plant-based meats are gaining popularity.

Cell-based food is currently recognized by the Food and Agriculture Organization of the United Nations (FAO) as a standardized term (FAO, 2023). It is receiving attention as a new technology that can meet the increasing demand for meat, while overcoming the limitations of traditional meat production methods. Livestock farming, especially cattle rearing, has a significant impact on the environment, health, and animal welfare (Ramani et al., 2021). In this context, cell-based food produced through *in vitro* cell cultivation has the potential to serve as a safe and efficient alternative to traditional slaughter-based meat production methods. Muscle is a critical tissue responsible for animal movement and body structure, and the development and differentiation of muscle cells play a pivotal role in muscle tissue formation (Mukund & Subramaniam, 2020).

The cells used in cell-based meat research are skeletal muscle satellite cell (SMSCs) located between the sarcolemma and basal lamina (Ding et al., 2017). These SMSCs are essential for muscle growth, repair, and regeneration and can influence changes in muscle conditions (Kim et al., 2022). The myogenic differentiation ability of SMSC depends primarily on the expression of Pax 3 and 7 genes and muscle regulatory factors (MRFs), including MyoD, Myf5, Myogenin, and MRF4 (Asfour et al., 2018). Sequential activation and inhibition of Pax3/7 and MRFs are essential for muscle-forming processes in muscle cells (Collins et al., 2009). Pax7 is expressed in all SMSC and is indispensable for postnatal maintenance and self-renewal (Seale et al., 2000). Myogenin regulates the development and differentiation of muscle cells, and is expressed during the formation of muscle tissue (Zhang et al., 2020). MRF4, also known as MYF6, is involved in the development of muscle cells, and regulates their differentiation and growth (Shirakawa et al., 2022). MHC1 is a protein related to muscle tissue contraction and is an important gene expressed when muscle cells differentiate, mature, and form muscle tissue (Kim et al., 2023).

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78 Korean cattle are divided into steers and cows, and differences exist between their meat
79 characteristics including taste (Cho et al., 2020). Steers are known to have relatively mild-tasting
80 meat, whereas cow meat has a rich, salty flavor (Gajaweera et al., 2020; Joo et al., 2017). Various
81 factors, such as age, breed, and sex, may lead to differences in the growth rate and genetic makeup of
82 SMSC in Hanwoo cattle (Kim et al., 2023). Previous studies mainly conducted sex-based comparative
83 analyses of human and mouse SMSC. In addition, a previous study comparing males (bulls and steers)
84 and females (heifers) showed that bulls had faster growth than heifers (Reyneke, 1976). Despite this
85 finding, there is still a lack of sex-based comparative analyses of SMSC. Therefore, this study aimed
86 to investigate the differences in muscle cell growth and differentiation between Hanwoo steers and
87 cows.
88

89 ## Materials and Methods

90 ### Materials

91 Dulbecco's Modified Eagle Medium-F12 (DMEM-F12), fetal bovine serum (FBS), horse
92 serum (HS), and antibiotic-antimycotics (AA) from Gibco (Thermo Fisher Scientific, Waltham, MA,
93 USA) and Dulbecco's phosphate-buffered saline (DPBS) were obtained from Welgene (Gyeongsan,
94 South Korea). For RNA extraction, cDNA synthesis, and quantitative real-time PCR, Trizol reagent
95 (Accuzol^{Tm} Total RNA Extraction Reagent, Bioneer Corporation, Seoul, Korea), diethylpyrocarbonate
96 (DEPC) water (Bioneer Corporation, Seoul, Korea), a cDNA transcription kit (AccuPower
97 CycleScript RT PreMix, Bioneer Corporation, Seoul, Korea), qPCR MasterMix (Bioneer Corporation,
98 Seoul, Korea), and nuclease-free water (Ambion® , Austin, TX, USA) were used.
99

100 ### Isolation and culture of bovine muscle satellite cells

101 33 months of Bovine muscle tissues (three steers and three cows) were purchased from
102 Farmstory Hannaeng Bio&Food Co., LTD (Chungbuk, South Korea). Animal sampling methods were
103 used for ethical approval. Muscles were obtained from the top round muscle and transport to the lab.
104 After that, tissues were rinsed with 70% ethanol and washed three times with DPBS. The tissues were
105 minced using a meat grinder and digested with 0.8 mg/mL Pronase (Sigma-Aldrich, St. Louis, MO,
106 USA) for 40 min at 37℃ with vortexing every 10 min. After incubation, the tubes were centrifuged at
107 1,200 ×g for 15 min and the pellets were resuspended in DMEM-F12 containing 10% FBS. The
108 supernatant was discarded, and DMEM-F12 containing 10% FBS was added to the tubes, mixed and
109 centrifuged at 300 ×g for 5 min. The supernatant was filtered through a 100 μm cell strainer, collected
110 in 50 mL tubes and centrifuged at 1,200 ×g for 15 min. The final step was collection in GM
111 containing 10% dimethyl sulfoxide until used. Isolated bovine SMSC were cultured in a T-25 flask
112 containing DMEM-F12, 10% FBS, and 1% AA. In passage 3, muscle satellite cells were isolated
113 using Magnetic-Activated Cell Sorting with CD29, a marker known for muscle cells. The experiment

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114 was conducted using the same method described in the previous study (Kim et al., 2023).
115

116 **Cell proliferation and doubling time**

117 The steers and cows of the bovine SMSC were seeded at 2×10^4cells/mL in a 6-well plate.
118 SMSC were cultured in growth medium (DMEM-F12+GlutaMax (Gibco, Gaithersburg, MD, USA) +
119 1% AA + 10% FBS). After 24 h, the growth medium was replaced with fresh medium, and the cell
120 number was determined. The medium was replaced with fresh medium every 48 h. Trypsinization for
121 cell counting was performed using 0.05% trypsin-EDTA (Gibco, Gaithersburg, MD, USA) after 1, 3,
122 5, and 7 days. Cell number was analyzed using a hemocytometer (Counting Chamber; Paul
123 Marienfeld GmbH & Co., Wöllerspfad, Germany). Measurements for each sample were taken in
124 triplicate and averaged daily.
125

126 **Myogenic cell differentiation**

127 The steers and cows of the bovine SMSC were seeded using 4×10^4cells/mL in a 6-well plate
128 in order to check the myogenic cell differentiation. SMSC were cultured in a growth medium
129 consisting of DMEM-F12/GlutaMax containing 1% AA (Gibco, Gaithersburg, USA) and 10% FBS
130 (GM; Gibco, Gaithersburg, USA). When the confluency of the cells reached 80%, the medium was
131 changed to a medium containing DMEM-F12, 1% AA, and 2% HS (DM; Gibco, Gaithersburg, USA)
132 and was replaced every 24 h. When SMSC started to differentiate, their morphology was analyzed.
133 Cell differentiation was evaluated at 24, 48, 72, and 96 h. For cell differentiation analysis, SMSC
134 were stained using May–Grünwald solution (Sigma Aldrich, USA) and Giemsa stain solution (Sigma
135 Aldrich, USA). After removing the medium, the cells were washed twice with DPBS, fixed with
136 100% methanol for 10 min, and methanol was subsequently removed. May-Grünwald solution was
137 added for 5 min, followed by dilution with distilled water. After removing the solution, Giemsa
138 staining solution (1:20) was then added for 20 min. Finally, the solution was removed, and the stained
139 cells were examined under a microscope (CKX53, OLYMPUS, Tokyo, Japan).
140

141 **Total RNA isolation and the cDNA synthesis**

142 Total RNA was isolated using TRIzol reagent (AccuzolTM Total RNA Extraction Reagent,
143 Bioneer Corporation, Seoul, Korea) according to the manufacturer's guidelines and previous protocol
144 (Kim et al. 2023). Next, 20 µL of DEPC water (Bioneer Corporation, Seoul, Korea) was added, and
145 the purity and concentration of RNA were measured using a Microplate Spectrophotometer
146 (Multiskan Sky, Thermo Fisher Scientific, USA) and a µDrop plate (µDrop™, Thermo Fisher
147 Scientific, USA). The total RNA concentration was determined to adjust to 1 µg of RNA. The RNA
148 samples were then reverse-transcribed into cDNA using a cDNA reverse transcription kit (AccuPower
149 CycleScript RT PreMix; Bioneer Corporation, Seoul, Korea) with a GeneAmp PCR System 9700

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150 (Applied Biosystems, Singapore). The machine was run based on the cDNA synthesis condition,
151 which consisted of the synthesis step at 45℃ for 60 min and the heat inactivation step at 95℃ for 5
152 min.
153
154 **Quantitative real-time PCR (qPCR)**
155 qPCR was performed on the cDNA using AccuPower® 2X Greenstar qPCR MasterMix
156 (Bioneer Corporation, Seoul, Korea) and a StepOnePlus Real-Time PCR system (Applied
157 Biosystems, Singapore) following the manufacturer's guidelines and a previous study (Kim et al.
158 2023). All the samples were recorded in triplicate, and the primer sequences for housekeeping and
159 target genes, as well as the cycling temperatures, are provided in Table 1 and 2. And the data was
160 used in order to analyze it using the ΔΔCt method. Calculation of ΔΔCt was conducted as follows.
161 First, the cycle threshold was obtained for each sample by qPCR. Second, in order to calculate the
162 ΔCt values, the Ct value of the housekeeping gene was subtracted from that of the target gene. Third,
163 the ΔΔCt values were obtained by subtracting the ΔCt value of the steer group as the control from the
164 ΔCt value of the cow group. Finally, the relative quantity value was obtained by calculation of $2^{-\Delta\Delta Ct}$.
165
166 **Statistical analysis**
167 All data were collected through experiments conducted in triplicate, and the results are presented
168 as the mean ± standard deviation. Statistical analysis was performed using two-way analysis of
169 variance in Prism 9.4.0 (GraphPad), and statistical significance was defined at a significance level of
170 *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
171

172 Results and Discussion

173
174 **Cell proliferation and doubling time**
175 Each muscle contains SMSC, which play a key role in muscle development, proliferation, metabolic
176 characteristics, and meat quality(Bazgir et al., 2016; Koike et al., 2022). SMSC, as the primary
177 proliferative components of skeletal muscles, exhibit self-renewal capabilities that are crucial for
178 muscle growth and repair. Moreover, the characteristics of muscle cells can vary depending on factors
179 such as sex, breed, and age (Kim et al., 2022). These variations may further influence the overall
180 metabolic characteristics and meat quality of the muscle. Approximately 35–60% of the total muscle
181 mass in livestock and fish is utilized for human food production. Among these, skeletal muscles
182 comprise approximately 90% muscle fibers and 10% connective and adipose tissues (Listrat et al., 2016).
183 The growth performance and body composition of cattle are influenced by sex. Comparative studies on
184 growth performance based on sex have revealed that bulls exhibit higher growth rates than steers and

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185 heifers (Reyneke, 1976). In addition, studies in mice have indicated a greater number of myofiber
186 SMSC in males than in females (Day et al., 2010). These findings confirmed the increased presence of
187 myofiber SMSC based on hormone levels associated with sex. To this end, we examined whether there
188 were sex-based differences in the growth rates of bovine muscle cells (Figure 1). The results confirmed
189 that steers exhibited higher cell growth rates than cows (Figure 2A,B). In the case of steers, cell counts
190 started at 2×10^4 and reached $6.93\pm0.251\times10^4$, $9.33\pm0.289\times10^4$, $10.83\pm0.285\times10^4$, and $13.5\pm0.5\times10^4$
191 during 7 days, while cows started at 2×10^4 and reached $4.56\pm0.115\times10^4$, $8.66\pm0.0.289\times10^4$, $9\pm$
192 0.284×10^4, and $11.3\pm0.763\times10^4$, respectively (Figure 2A). As indicated in Figure 2B, when calculating
193 the doubling time using cell counts, significant differences were observed between steers and cows at
194 24, 120, and 168 h ($p<0.0001$). To confirm this finding, examination of cell images revealed that steers
195 exhibited a higher cell growth rate and lower doubling time than cows, and this difference in muscle
196 cell growth rate was also significantly influenced by sex.
197

198 **Myogenic cell differentiation**

199 According to Verdijk et al., differences in muscle growth and differentiation rates between
200 male and female muscle cells were observed (Verdijk et al., 2014). Mouse experiments regarding sex-
201 specific differentiation rates and growth indicated that male mice demonstrated faster differentiation
202 and growth rates than female mice (Neal et al., 2012). This was confirmed to affect the differentiation
203 rates based on doubling time. Moreover, Rosa-Caldwell and Greene stated that males tend to exhibit
204 higher levels of testosterone, a transcription factor that enhances myogenesis and muscle protein
205 synthesis (Rosa-Caldwell & Greene, 2019). Additionally, the administration of testosterone to animals
206 or individuals with low testosterone levels, such as elderly male rodents, results in increased muscle
207 mass (Bhasin et al., 1997; Rosa-Caldwell & Greene, 2019; Sinha et al., 2014). Based on previous studies,
208 we investigated whether there are sex-based differences in the muscle satellite cell differentiation rate
209 in cattle. For differentiation, the cell culture medium was replaced with DM when the cells were over
210 80% confluent, and the time point was set at 0 h. As shown in Figure 3, the differentiation results
211 between steers and cows revealed that steers had already formed myotubes after 24 h, whereas cows
212 showed no differentiation. After 48 h, steers continued to undergo differentiation, while cows had not
213 yet initiated the process. By 72 h, the differentiation of cow SMSC had begun, and steers formed a
214 greater number of myotubes. After 96 h, both cow and steer muscle cells exhibited a gradual increase
215 in differentiation. Figure 4 displays the cell fusion index results for steer and cow SMSC. The steers
216 exhibited fusion indices of 5.6% at 48 h, 21.66% at 72 h, and 33.3% at 96 h ($p<0.001$). In contrast, cows
217 showed a fusion index of 0% at 48 h, 16.33% at 72 h, and 23% at 96 h ($p<0.001$). This differentiation
218 pattern also exhibited similarities with proliferation, indicating sex-based differences in differentiation

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(Verdijk et al., 2014).

Genetic analysis

Skeletal muscles possess a high regenerative capacity, allowing them to adapt to physiological demands such as growth or exercise (Murach et al., 2021). In the quiescent state, SMSC are activated, expanded, and undergo differentiation following a program similar to fetal muscle formation. Muscle growth and differentiation occur in four stages: progenitor cells, myoblasts, myotubes, and myofibers (Choi et al., 2021). In the early progenitor stage, the Pax7 gene is expressed, playing a critical role in muscle growth and regeneration (Cosgrove et al., 2009). Activated SMSC are also involved in the repair of damaged muscles and the generation of new muscle fibers. Subsequently, in the myoblast stage, muscles grow until they differentiate. At this stage, Pax7 expression decreases and the myogenin gene begins to be expressed. Myogenin is crucial for muscle cell maturation, myotube formation, the promotion of muscle cell differentiation, and protein production (Zammit, 2017). During the transition from myocyte to myotube, MRF4 expression begins (Zammit, 2017). MRF4, another muscle differentiation gene, contributes to muscle cell maturation and myofiber formation (Kassar-Duchossoy et al., 2004). Activated SMSC play a pivotal role in regulating cell proliferation and differentiation to maintain muscle formation and function. Subsequently, they evolve into myofibers in which MHC1 is highly expressed. MHC1 is a protein associated with muscle contraction that enables muscle fibers to contract (Robelin et al., 1993). It is a key muscle protein that enables contraction within muscle cells. These genes cooperate during muscle development and maturation in order to regulate and maintain muscle formation and function. To understand the roles of these genes, we conducted real-time PCR analysis of the growth and differentiation of steers and cows. On the 5th day of growth in the gene expression comparison analysis (Figure 5A), we observed that Pax7 was expressed at higher levels in steers than in cows ($p<0.0001$). MyoG, MRF4, and MHC1 showed a tendency for higher expression levels in steers than in cows; however, these differences were not statistically significant. In the gene expression comparison analysis on the 3rd day of differentiation (Figure 5B), Pax7 showed lower expression levels than those during cell growth. MyoG, MRF4, and MHC1 exhibited higher expression levels in steers than in cows ($p<0.001$, $p<0.01$, and $p<0.0001$, respectively). These results support those of the experiments related to cell growth and differentiation, indicating that steers with high Pax7 expression have a higher cell count and lower doubling time for cell growth than cows. Additionally, during differentiation, steers with a higher expression of MyoG, MRF4, and MHC1 showed a higher fusion index than cows.

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Conclusion

In summary, our study revealed significant differences in cellular proliferation and differentiation processes within the muscle tissue of Hanwoo steers and cows. Steers exhibited higher growth and fusion rates, reflected in the increased Pax7 expression during growth and elevated MyoG, MRF4, and MHC1 expression during differentiation, compared to cows ($p<0.0001$). These findings mirror observations in human muscle cells, indicating accelerated growth and differentiation, which are crucial for meat production. These investigations contribute to a better understanding of muscle tissue development and offer insights into the modulation of meat quality in the meat production industry.

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Tables and Figures

Table 1. qPCR reaction condition.

Stage	Step		Temp (°C)	Time
Holding	1. Initial denaturation		95	5 min
Cycling (Repeat 40 cycles)	2. Denaturation		95	30 sec
	3. Annealing	GAPDH	55	30 sec
		PAX7	53	
		MRF4	56	
		MHC1	54	
	4. Extension		72	90 sec
Melt curve	5. Final extension		95	15 sec
			60	1 min
			95	15 sec
	6. Hold		4	∞

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381 Table 2. The primer sequences used for quantitative real-time PCR

Gene	Primer sequence (5′-3′)	Reference
GAPDH	F: CTGGCAAAGTGGATGTTGTC	(Li et al., 2022)
	R: GCATCACCCCACTTGATGTTG	
PAX7	F: TGCCCTCAGTGAGTTCGATT	(Li et al., 2022)
	R: CGGGTTCTGACTCCACATCT	
MyoG	F: AGAAGGTGAATGAAGCCTTCGA	(S et al., 2019)
	R: GCAGGCGCTCTATGTACTGGAT	
MRF4	F: GGTGGACCCCTTCAGCTACAG	(Shibata et al., 2022)
	R: TGCTTGTCCCTCCTTCCTTGG	
MHC1	F: CCCACTTCTCCCTGATCCACTAC	(JS et al., 2020)
	R: TTGAGCGGGTCTTTGTTTTTCT	

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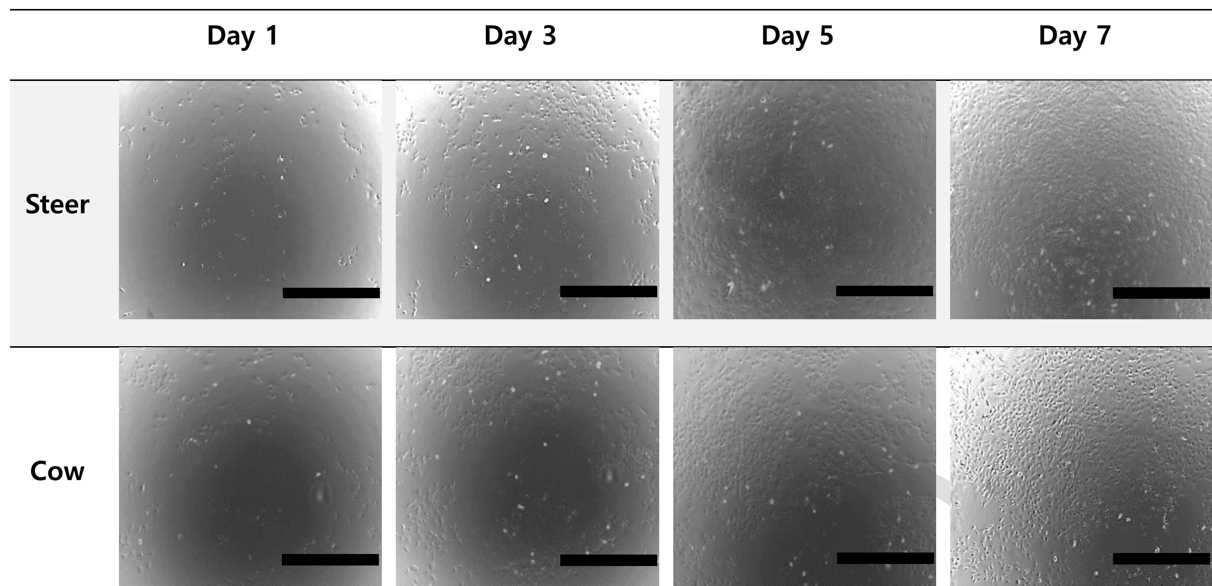

384 Figure 1. Cell morphology of steer and cow of bovine muscle satellite cell proliferation. (Scale bar= 1000μm)

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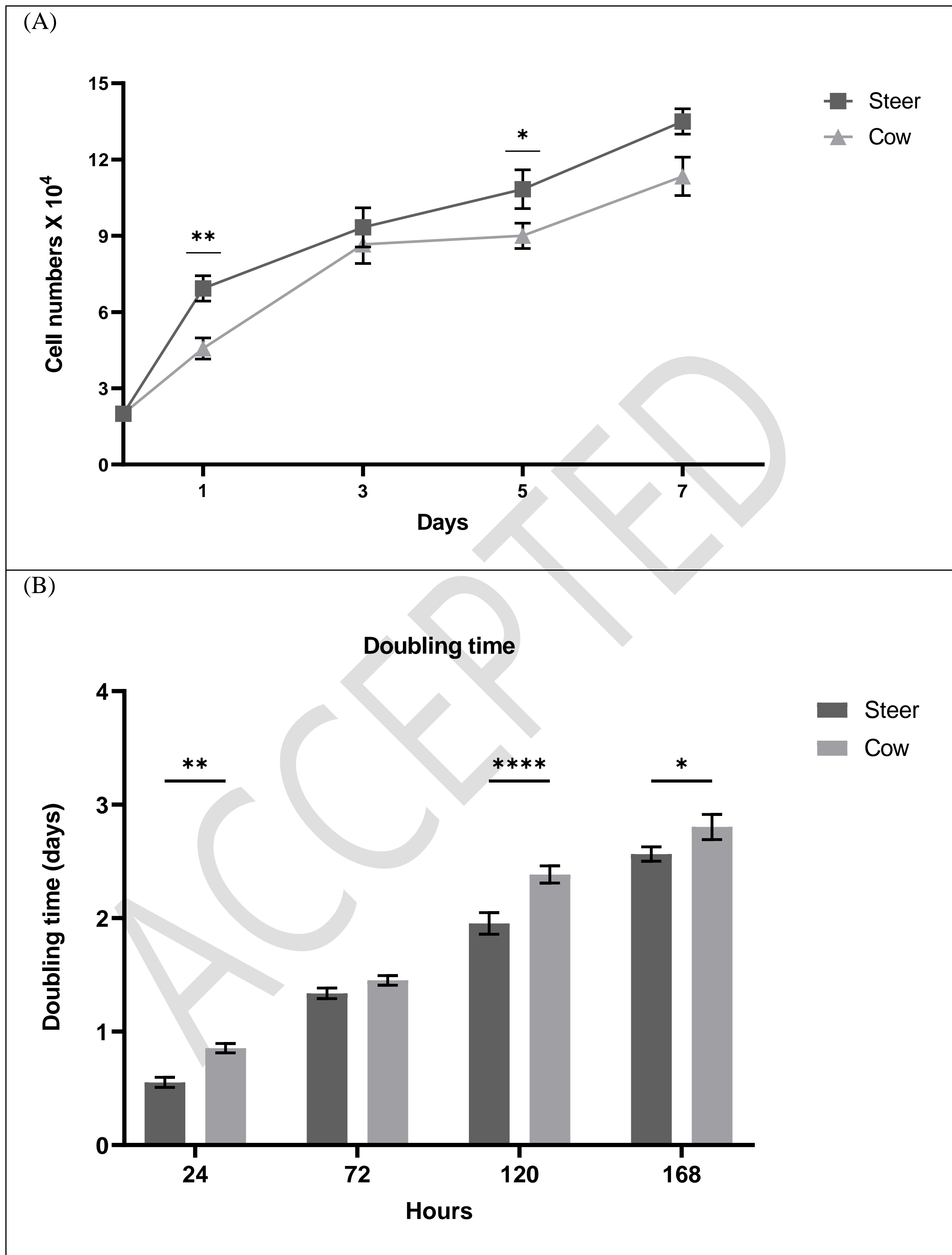

386 Figure 2. (A) Changes in cell number between steer and cow during 7 days. (B) Cell doubling time between
387 steer and cow during 7 days. (n=3 in each group), Results were expressed as mean ± standard deviation,
388 (*p<0.05, **p<0.01, ****p<0.0001).

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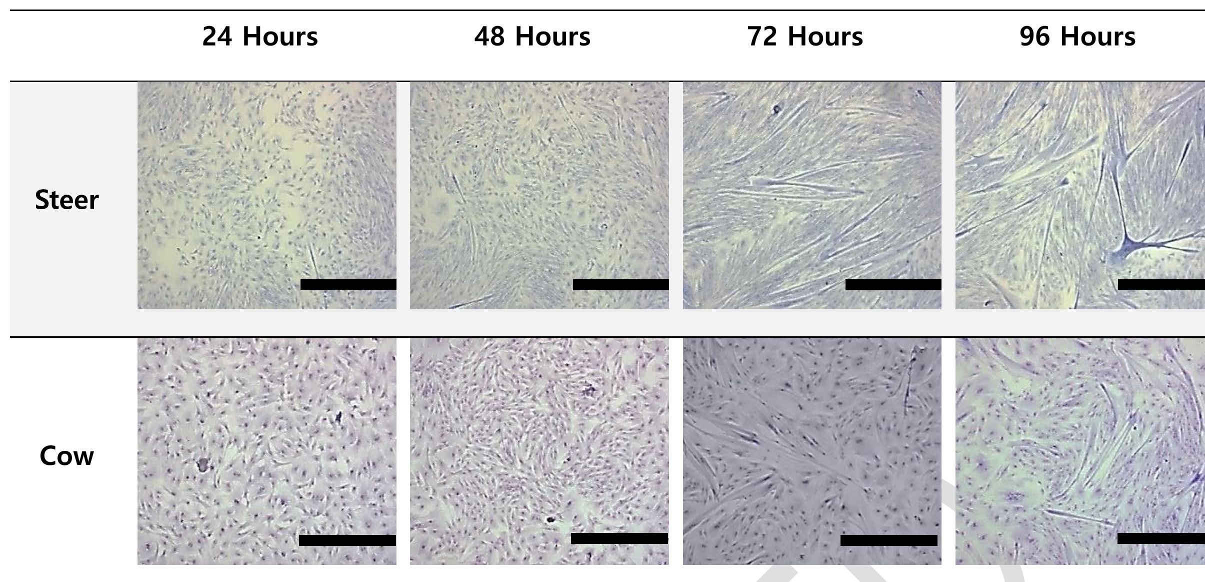

Figure 3. Differentiation of steer and cow muscle satellite cells. (Scale bar= 1000μm)

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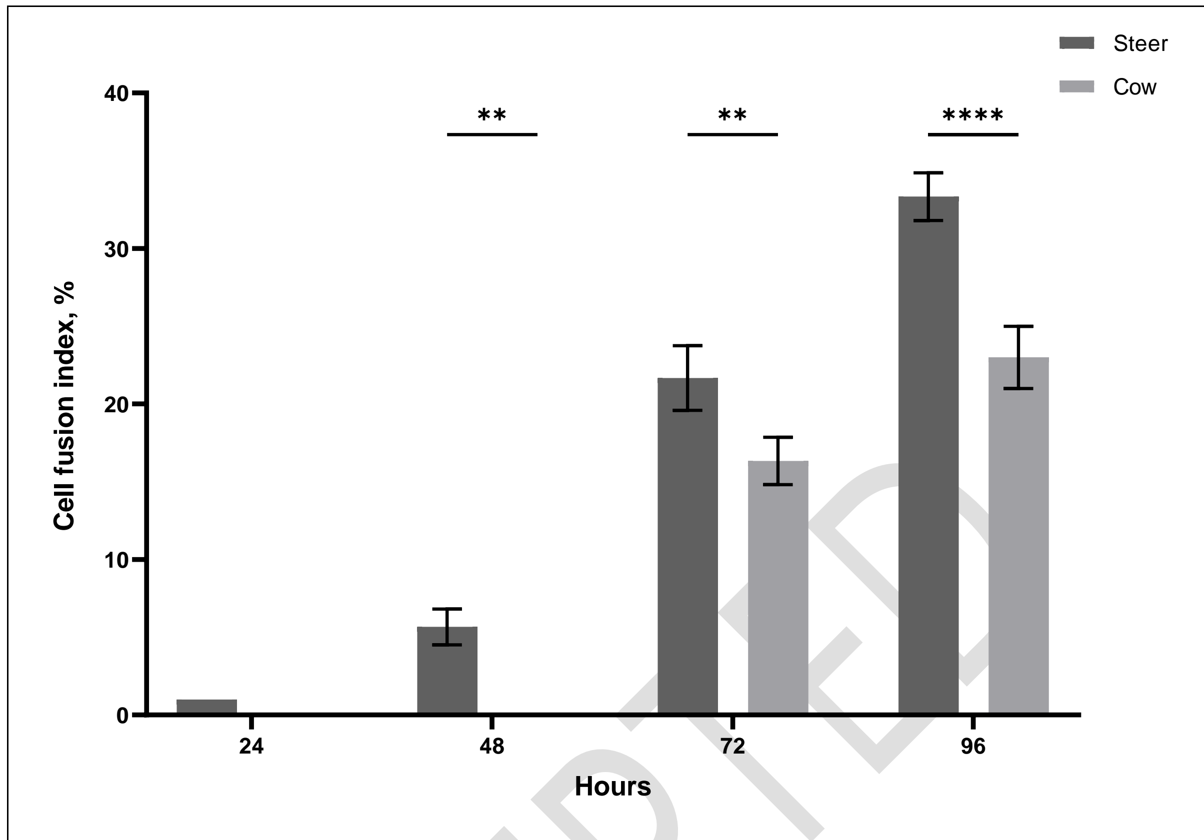

Figure 4. Cell fusion rate after differentiation of steer and cow muscle satellite cell. Results were expressed as mean ± standard deviation, p<0.05 **p<0.001, ****p<0.0001, (n=3).

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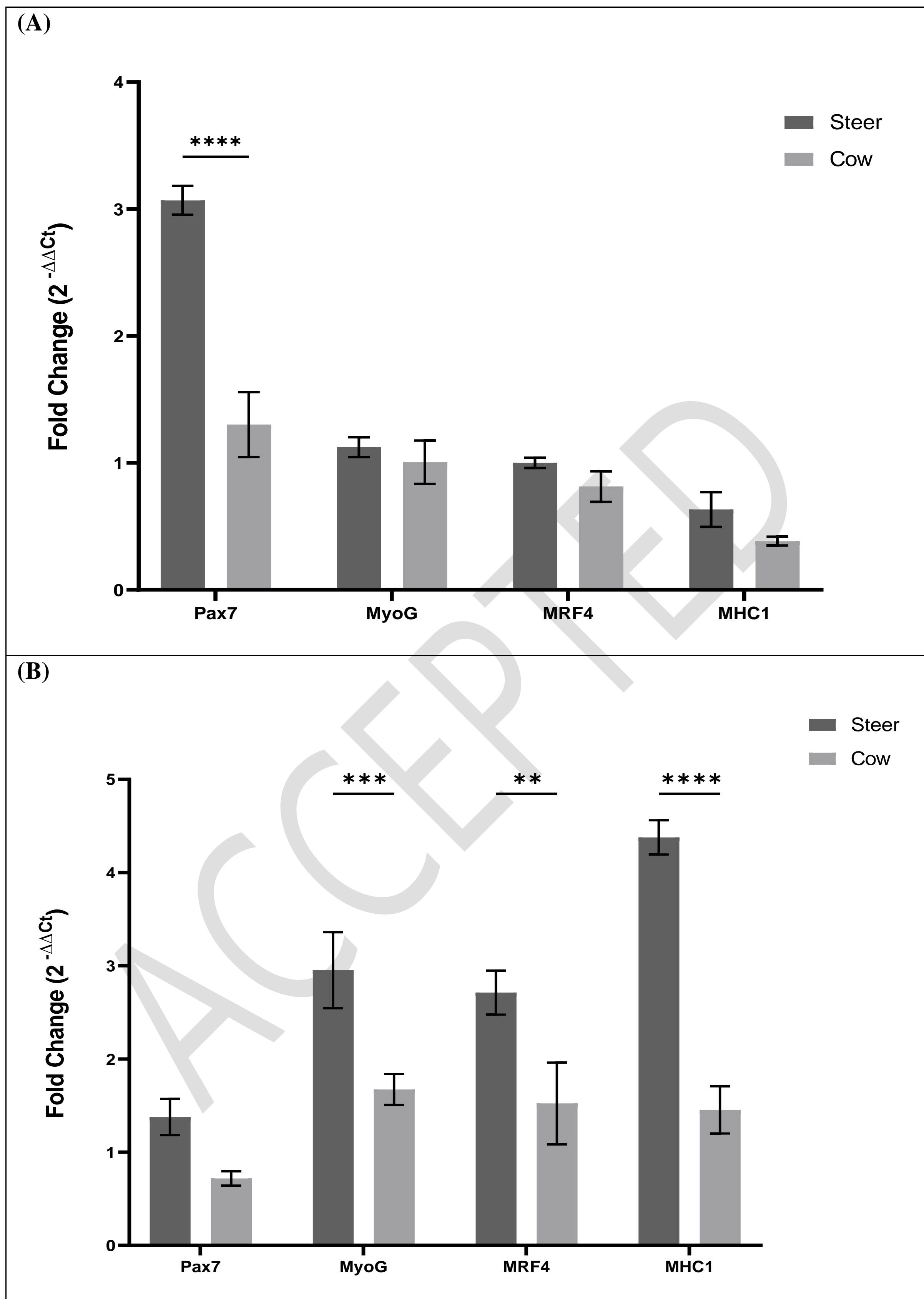

Figure 5. Relative gene expression of muscle satellite cells isolated from steer and cow was analyzed by real-time qPCR. (A) Gene expression pattern of steer and cow muscle satellite cells during proliferation. (B) Gene expression pattern of steer and cow muscle satellite cells after fully differentiated. Results were shown as mean ± standard deviation (n=3 in each group, **p<0.01, ***p<0<0.001, ****p<0.0001)

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