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Effect of glucose and lysine supplementation on myogenic and adipogenic gene expression in muscle satellite cells isolated from Hanwoo with different genotypes of PLAG1: Implications for cell-based food production

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Research on Hanwoo cattle has focused on the pleomorphic adenoma gene (PLAG) family, vital for traits like growth and carcass quality. Single nucleotide polymorphisms (SNPs) within this gene family profoundly impact economic traits. At the cellular level, energy and protein sources, notably glucose and lysine, crucially regulate muscle satellite cell (MSC) growth and differentiation.

This study delved into how varied glucose and lysine levels affect gene expression patterns in Hanwoo MSC. MSC from 9 Hanwoo, aged 29–36 months, categorized into 3 PLAG1 genotypes (GG, GC, CC), were treated with six combinations of glucose (5.5 and 25 mM) and lysine (2, 4, and 8 mM). Analysis of myogenic and adipogenic genes linked to meat quality and quantity ensued.

The GG genotype displayed superior dressed percentage, yield grade, and marbling score, hinting at genotype-associated carcass characteristic disparities. In cell culture, gene expression generally rose with lysine addition to high glucose in the GG group. Contrarily, significant differences across all treatments in the GC genotype suggested distinct responses. Significant effects of genotype, glucose, and lysine on cell proliferation-related gene expression were noted. Highest mRNA expression for MyoD, MyoG, and FASN occurred in the CC genotype, while Myf5 and Pax7 expression peaked in the GG genotype. Glucose significantly influenced Pax7 and FASN expression, while lysine positively impacted MyoD and MyoG genes. Notable interactions, especially in Genotype × Lysine, influenced MyoD, Myf5, and Pax7 expression, highlighting complex relationships in cell proliferation. Regarding cell differentiation, Pax7 expression was highest in PLAG1 GG type. High glucose prompted wider myotubes, while lower lysine concentrations slightly favored cell differentiation. Correspondingly, MyoG expression decreased with higher lysine levels.

This study furnishes insights into lysine and glucose supplementation effects on bovine MSC proliferation and differentiation, considering PLAG1 genotype influence. It offers valuable data for beef production system establishment and optimizing cell-based food production.

1. Introduction

Hanwoo is native cattle of Korea and well known for its high quality meat production capacity with better marbling scores, relatively thin muscle fibers, and less connective tissues (Koh et al., 2019; Song et al., 2020; Hwang et al., 2010). Recent research has also focused on optimizing meat production efficiency by identifying the correlation between environment and genetic makeups of cattle. In addition, studies have pivoted towards optimizing meat production, with a particular emphasis on validating the efficacy of specific genetic loci (Lee et al., 2013; Song and Hwang, 2023). For instance, results have underscored the significant role of the Pleomorphic adenoma gene 1 (PLAG1) in

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myoblast proliferation and expression, tightly linked to cattle growth (Wang et al., 2022a; Karim et al., 2011). Recently, the significant influence of PLAG1 mutation in alterations of body size observed in Bos taurus breed represents one of the pioneering instances of a genomic sweep in livestock, instigated by selection of a multifaceted trait (Utsunomiya et al., 2017; Littlejohn et al., 2012). Association of PLAG1 SNP on carcass weight and fatty acid composition in beef produced from Hanwoo steers was identified (Li et al., 2020). Expression level of PLAG1 gene was greater in fetuses than in adult cow (Xu et al., 2018). 19bp deletion of the PLAG1 gene negatively affected the growth of bovine (Hou et al., 2020; Kim et al., 2017).

The development and maturation of muscle cells is regulated by the intricate interplay of numerous genes and regulatory factors, such as Muscle Regulatory Factors (MRFs) including Myogenic Factor 5 (Myf5), Myogenic Differentiation 1 (MyoD), Myogenin (MyoG), and Paired box 7 (Pax7), which play crucial roles in muscle cell development and maturation (Park et al., 2022; Kim et al., 2023a). Pax7 is known as a gene involved in muscle growth and regeneration (Rahman et al., 2023). Myf5 is recognized as a gene involved in the early differentiation of muscle cells, while MyoD and Myf5 are implicated in promoting muscle cell differentiation (Conerly et al., 2016). Additionally, MyoG is associated with muscle cell maturation and the formation of muscle fibers (Ganassi et al., 2020). The fatty acid synthase (FASN), playing important roles in fatty acid synthesis, also affects the fatty acid content in beef (Song et al., 2020; Kim et al., 2023b).

Exogenous amino acids serve as primary contributors to cell growth and development. Among these, lysine plays a particularly crucial role in the proliferation of muscle cells and protein biosynthesis in cattle (Lin et al., 2018; Morales et al., 2015). Supplementation of lysine in the diet of dairy cows has been shown to elevate plasma concentration, resulting in increased milk volume and milk protein content (Rogers et al., 1989; Nishimura et al., 2012). Additionally, quality of dietary protein significantly influences mammary protein synthesis, as evidenced by cows fed rumen-protected methionine and lysine feed exhibiting greater milk yield and reduced risk of mastitis compared to control cows (Abreu et al., 2023).

Another important exogenous nutrient glucose is essential energy source for cell proliferation and differentiation (Yucel et al., 2019). Recent studies have demonstrated a complicated regulatory roles of glucose in cell biology showing that the proliferation of rat muscle satellite cells is more active in a 2 mM Low glucose medium than in a 19 mM High glucose medium (Furuichi et al., 2021). Similarly, pig muscle satellite cells cultured in a high glucose medium (25 mM) showed enhanced expression levels of key genes involved in differentiation, alongside improved protein synthesis (Yue et al., 2010). Additionally, cell proliferation was improved when higher levels of glucose (10 mM–20 mM) were supplemented to primary bovine mammary epithelial cells (BMEC) compared to lower glucose (2.5 mM) was added ($p < 0.05$) (Zhao et al., 2012). These findings suggest that low glucose medium enhances cell proliferation, while high glucose medium improves gene expression levels in the differentiation of muscle satellite cells.

Results from the current study demonstrate that administration of Hanwoo muscle satellite cells with various levels of lysine and glucose affects the cell metabolism, morphology, and developmental characteristics with related gene expression. Based on our hypothesis, glucose and lysine as main energy and building block, supplementation of glucose and lysine may affect the growth and development of skeletal muscle cells isolated from cattle and their effect may vary depending on PLAG genotype, the current study aimed to analyze the growth and differentiation rate and related gene expression of muscle satellite cells treated with varying concentration of glucose and lysine as energy and protein sources. Results may provide useful information for development of cell-based food production.

2. Materials and methods

2.1. Material

Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12), Fetal Bovine Serum (FBS), and PBS were purchased from WELGENE (Gyeongsan, Korea). Dulbecco's Modified Eagle's Medium (DMEM), and Horse Serum (HS) were purchased from Sigma-Aldrich (St Louis, MO, USA), and cDNA kit and AccuPower® RocketScript™ Cycle RT PreMix & Master Mix were purchased from Bioneer (Daejeon, Korea). The MTT Assay kit was purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Cell isolation

Rump muscle tissues were collected from nine Hanwoo steers and heifers (29–36 months) were obtained from Farm Story Hannaeong Bio&Food Co., LTD (Chungbuk, South Korea). After slaughtered, the samples were placed in PBS with 3% Antibiotic-Antimycotic (A.A; Gibco, Gaithersburg, MD, USA) solution and refrigerated at 4 °C until cell isolation. The collected muscle tissue was finely ground three times with a grinder, and then 10 g of sample and 30 mL of pronase solution (Sigma P5147, St. Louis, MO, USA) were placed in a 50 mL conical tube. The sample was placed in a 37 °C water bath for 50 min and shaken every 10 min to mix. Afterwards, the sample was removed from the water bath and centrifuged at 1500×*g* for 4 min. After centrifugation is completed, discard the pronase solution, add 3 mL of DPBS with 1% A.A solution, and shake. Then, centrifugation was performed at 400×*g* for 10 min, and the PBS solution was removed. After repeating the previous process three times, add 30 mL conditioning medium and filter to filter out the pellet. Samples were centrifuged at 1500×*g* for 10 min, and conditioning medium was discarded. After adding freezing medium and pipetting, freeze cells at 1 mL per cryovial.

2.3. Genotype analysis

The genotype was analyzed by collecting Korean beef rump, and a Genomic DNA kit (Macrogen, MG-P-011-100) was used to extract Genomic DNA. PLAG1 PCR was performed at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 40 s, and this process was repeated 40 times. Then it was maintained at 72 °C for 5 min. After PCR was completed, electrophoresis was performed on a 1% agarose gel to check whether DNA was amplified. PCR Primer Sequence is shown in Table 1. When PCR product was generated after electrophoresis, Restriction Fragment Length Polymorphism (RFLP) was performed. The enzyme used was *Hph*I (Biolabs), and 3.9 μL of DEPC Water, 1 μL buffer, and 0.2 μL Enzyme were mixed and incubated overnight in an incubator at 37 °C.

2.4. Cell culture

Hanwoo bovine muscle satellite cells were cultured, and the medium and cell information used in this experiment are showed in Table 2. Three repetitions were performed with 3 animals per PLAG1 genotype (GC, GG, CC). A 100 mm diameter culture dish was used for cell cultured and incubated at 37 °C. The medium was changed once every three days. When the cell reached to 80% confluency, the subculture was

Table 1
Primer sequence for PLAG1 SNP (g. 25,003,338 C > G).

Primer name	Primer sequence (5′-3′)	Product size	Annealing TM	Restriction Enzyme
PLAG1_F	CAA GGG CTC AAC GTA GG	374bp	53 °C	*Hph*I
PLAG1_R	TGT TTC AAG TGC CAT TAG AGG			

Table 2
Cell culture media components.

	Components	
	Glucose	Lysine
Control 1[a]	High glucose 25 mM	–
Control 2[b]	Low glucose 5.5 mM	–
Treatment 1[c]	High glucose 25 mM	2 mM
Treatment 2[d]	High glucose 25 mM	4 mM
Treatment 3[e]	High glucose 25 mM	8 mM
Treatment 4[f]	Low glucose 5.5 mM	2 mM
Treatment 5[g]	Low glucose 5.5 mM	4 mM
Treatment 6[h]	Low glucose 5.5 mM	8 mM

[a] Control 1: C1.
[b] Control 2: C2.
[c] Treatment 1: T1.
[d] Treatment 2: T2.
[e] Treatment 3: T3.
[f] Treatment 4: T4.
[g] Treatment 5: T5.
[h] Treatment 6: T6.

performed.

2.5. Cell proliferation assay

Bovine muscle satellite cells were seeded into a 6-well plate at 5×10^4 cells/mL. The dispensed cells were cultured in growth media containing with DMEM/F12 medium with FBS 10% and 1% A.A, and 1% Kanamycin (GM) in an incubator for 24 h at 37 °C and 5% CO_2. After 72 h, the medium was replaced with medium for the experimental and control groups, respectively, and cultured for 96 h. Proliferated cells were observed using a microscope. Cells were classified into 3 groups based on their genotypes (GG, GC, CC) treated with different levels of glucose (G7021, Sigma Aldrich, USA; low 5.5 mM, high 25 mM), and lysine (L5501, Sigma Aldrich, USA; 0 mM, 2 mM, 4 mM, 8 mM). Cell number was analyzed by using a hemocytometer.

2.6. Cell differentiation assay

A 6-well plate was seeded at 5×10^4 cells/mL and cultured for 72 h at 37 °C and 5% CO_2 using GM. When the cells reached to 70%, the medium containing 2% HS for each treatment group was replaced and cultured for 96 h to differentiate.

After 96 h, the cells were stained with Giemsa stain solution (Sigma-Aldrich). After removing all existing media and washing twice with DPBS, and 500 μL of methanol was added to each well. After shaking for 2–3 min, methanol was removed, and air-drying was performed for 10 min. Then, 0.5 mL of MG Stain was added to each well, mixed well, and left for 1 min and 30 s. The reagents were removed, and 2 mL of Giemsa staining solution (× 20) was added to each well, shaken lightly, and left for 20 min. After 20 min, all reagents were removed, and each well was washed twice with 2 mL of sterile distilled water. Air-dry for 15 min, and myotubes staining was observed under a microscope (Eclipse TS2, Nikon, Japan).

2.7. MitoTracker staining

A 6-well plate was seeded at 5×10^4 cells/mL and cultured for 72 h at 37 °C and 5% CO_2 using GM. When the cells reached to 70%, the medium containing 2% HS for each treatment group was replaced and cultured for 96 h to differentiate. After that, the medium was removed, and 0.5 mL of the new medium was replaced in each well. 0.13 μL of 50 nM Mito Tracker Red CMX-ROS (Danver, MA, USA) and 0.5 μL of 1 μM Hoechst 33,342 dye (San Jose, CA, USA) were added to each well and incubated at 37 °C for 30 min. After incubation, Mito Tracker Red CMX-ROS and Hoechst 33,342 dye were removed and washed twice using a cell medium. The cells were fixed with 4% formaldehyde, incubated at 37 °C for 15 min, and washed twice with DPBS. Stained cells were observed in myotubes using a fluorescence microscope (Eclipse TS2, Nikon, Japan).

2.8. Gene expression analysis

Each medium was replaced with the experimental and control groups, and RNA extraction was performed 96 h later. After removing the existing medium from the 6-well plate, the plate was washed twice with 2 mL of PBS for each well. 1 mL of Trizol (Invitrogen, Waltham, MA, USA) and 200 μL of Chloroform were added to each well. RNA was extracted according to the protocol provided by the manufacturer. 20 μL of DEPC Water was added to the extracted RNA, and RNA concentration and purity were measured using Nano drop (KLab, Daejeon, Korea). The synthesized cDNA was subjected to RT-PCR using AccuPower® 2X Greenstar qPCR MasterMix (Bioneer, Seoul, Korea) to measure gene expression level. RT-PCR was performed according to the protocol provided by the manufacturer. GAPDH was used as a house keeping gene, and RT-PCR was monitored using the CFX ConnectTM Real-Time System (Bio-Rad). Primers used in the experiment are listed in Table 3.

To analyze the data, the ΔΔCt method was used. First, the ΔCt values were calculated by subtracting the Ct value of the housekeeping gene from the Ct value of the target gene. Then, the ΔΔCt values were determined by subtracting the ΔCt value of the control group from the ΔCt value of the treatment group. Finally, the relative gene expression levels were calculated using formula $2^{-\Delta\Delta Ct}$.

2.9. Cell viability assay (MTT assay)

A 96 well-plate was seeded at 5×10^3 cells/mL and cultured in DMEM/F12 medium at 37 °C and 5% CO_2 for 24 h. After 24 h, each treatment group was replaced with growth medium and cultured for 72 h. Then, the absorbance of cells was analyzed at 540 nm using the cell viability assay ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); MTT Assay kit). Cell viability was measured according to the manufacturer's protocol. The MTT Assay changes color from yellow to purple, and the higher the metabolic activity of the cell, change to deep purple. Cell viability calculation is as follows. (OD treated – OD blank)/(OD control – OD blank) wells × 100.

2.10. Statistical analysis

The results of this experiment were statistically analyzed using

Table 3
Forward and reverse primers for real-time PCR for specific gene mRNA.

Gene Name	Accession No.		Primer Sequence (5′-3′)
GAPDH[a]	NM_001034034	Forward	CACCACCATGGAGAAGGCCG
		Reverse	GAACACGGAAGGCCATGCCA
MyoD[b]	NM_001040478.2	Forward	TTTGCCAGAGCAGGAGCCCCTC
		Reverse	TTCGAACACCTGAGCGAGCGC
MyoG[c]	NM_001111325.1	Forward	CCGTGGGCGTGTAAGGTGTG
		Reverse	CCTCTGGTTGGGGTTGAGCAG
Myf5[d]	NM_174116.1	Forward	TGGCTGCTTTCGGGGCTCAC
		Reverse	GGTTGACCTTCTTCAGGCGTCTCC
Pax7[e]	XM_019984705.1	Forward	TGGTTCAGTAACCGCCGTGCC
		Reverse	TGCCCCCGTCTTGGGAGATAGTAG
FASN[f]	XM_027517867.1	Forward	ACCTTGACACGGCTCAACTC
		Reverse	CACTGTGGCCATAGGTGGG

[a] Glyceraldehyde 3-phosphate dehydrogenase.
[b] Myogenic Differentiation 1.
[c] Myogenin.
[d] Myogenic Factor 5.
[e] Paired box 7.
[f] Fatty acid synthase.

GraphPad Prism 10.1.10 to explain the effect of the interaction of genotype, glucose, and lysine on proliferation and differentiation, and related gene expression of Hanwoo muscle-derived satellite cells. Possible correlations between main effects were analyzed using 2-way-ANOVA and 3-way-ANOVA, and significant differences were considered at a threshold of $p < 0.05$.

3. Results

3.1. Genotype analysis

The genotype analysis results (GG, GC, CC) indicated that there were no significant differences between each genotype (Fig. 1). While the dressed weight, dressed percentage, yield grade, and marbling score by genotype did not show statistical significance, the GG type exhibited numerically higher values for dressed percentage, yield grade, and marbling score. According to Littlejohn et al. (2012), although the PLAG1 genotype is associated with growth rate and body weight, it does not significantly correlate with feed intake and other traits (Littlejohn et al., 2012). Consistent with this, our results also indicated that there was no significant differences between genotypes but it has the numerically higher in the GG type. Moreover, the PLAG1 GC type exhibited the highest dressed weight (Table 4).

3.2. Gene expression level according to treatment group by PLAG1 genotype in Hanwoo muscle satellite cell proliferation

3.2.1. Gene expression by treatment in PLAG1 GG type

The PLAG1 GG type, there was no significant difference in MyoD, but gene expression tended to increase as lysine was added to high glucose, and gene expression tended to decrease as lysine was added to low glucose. T3 had the highest gene expression level at 2.34, and T6 had the lowest gene expression level at 0.94. There was a significant difference in MyoG ($p = 0.0048$), and the gene expression level of T1 was the highest at 1.62 and there was a significant difference between T5 and T6. There was no significant difference in mRNA expression levels between treatment groups for Myf5 and FASN (Table 5).

3.2.2. Gene expression by treatment in PLAG1 GC type

In PLAG1 GC type, MyoD ($p < 0.0001$), the gene expression level of T6, which added 8 mM Lysine to Low glucose, was the highest at 15.44, and showed significant differences in all treatments. There was a highly significant difference with MyoG ($p < 0.0001$) and Pax7 ($p < 0.0001$). For Myf5, T5, which added 8 mM Lysine to low glucose, had the lowest gene expression level of 0.58, and C2, which had a high gene expression level, had 1.67, which was about three times that of T5. There was no significance between FASN treatment groups (Table 6).

3.2.3. Gene expression by treatment in PLAG1 CC type

In PLAG1 CC, MyoD ($p < 0.0001$), MyoG ($p < 0.0001$), Myf5 ($p = 0.0008$), and FASN ($p = 0.0003$) showed highly significant differences between treatments. There was no significant difference in Pax7, but gene expression tended to decrease as lysine was added (Table 7).

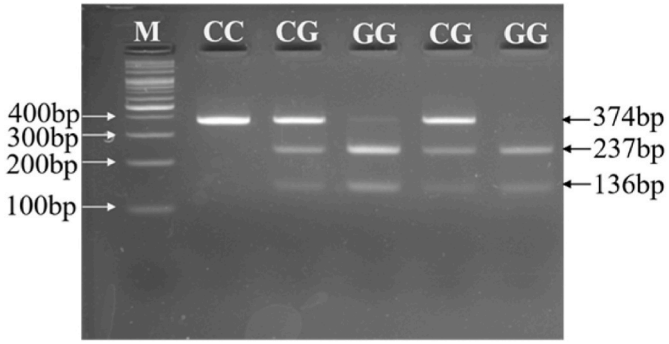

Fig. 1. PCR-RFLP analysis of PLAG1 g. 25,003,338 (3′UTR) C > G.

Table 4
Carcass yield and meat quality traits of Hanwoo based on PLAG1 genotype.

	PLAG1 GG	PLAG1 GC	PLAG1 CC
Carcass yield traits			
Dressed weight, kg	483.33 ± 38.73	507.00 ± 74.08	452.00 ± 91.03
Dressed percentage, %	62.11 ± 1.52	60.14 ± 0.75	61.21 ± 2.06
Yield grade [a]	2.22 ± 0.58	1.67 ± 0.58	2.00 ± 1.00
Quality traits			
Marbling score [b]	4.67 ± 0.58	4.67 ± 0.58	3.67 ± 0.58

[a] Yield grade: Grade A = 3~grade C = 1.
[b] Marbling score: Grade 1^{++} = 5~Grade 3 = 1.

3.2.4. Main effects of genotype, glucose, and lysine on proliferation

The genotype (GG, GC, CC) main effects were significant for MyoD ($p = 0.007$), MyoG ($p = 0.041$), Myf5 ($p = 0.016$), Pax7 ($p < 0.0001$), and FASN ($p = 0.038$) gene expression. The mRNA expression levels for MyoD, MyoG, and FASN were highest in the PLAG1 CC type. Gene expression levels for Myf5 and Pax7 were highest in the PLAG1 GG type. Glucose main effects were not significantly different in MyoD, MyoG, and Myf5, but Pax7 ($p < 0.001$) and FASN ($p = 0.0038$) gene expression were significant differences. Lysine treatment positively affected the expression of the MyoD ($p < 0.0001$) and MyoG ($p = 0.0019$) genes. There were no significant differences in Myf5, Pax7, and FASN, but gene expression levels tended to increase in the treatment group with Lysine than in the control group without Lysine (Table 8).

3.3. Gene expression level according to treatment group by PLAG1 genotype in Hanwoo muscle-derived cell differentiation

3.3.1. Gene expression by treatment in PLAG1 GG type

The PLAG1 GG type, there was no significant difference in MyoD, but gene expression tended to increase as lysine was added to high glucose. MyoG ($p = 0.0118$) showed a significant difference, and the relative gene expression level of C2, which did not add lysine to low glucose, was the highest at 3.06. C2 was significantly different from T2 and T6, meaning that the mRNA expression level decreased as lysine was added. Myf5 was not significant between the control and treatment groups, but the gene expression level tended to increase as lysine was added to high and low glucose. Pax7 showed a highly significant difference at ($p < 0.0001$), and the relative gene expression level was highest at 54.88 in T6, in which 8 mM Lysine was added to low glucose. This showed a gene expression level approximately 54 times higher than that of C1. T5 and T6 were significant with all treatments. FASN was significant for C1 and T6 ($p = 0.0088$), and the mRNA expression level tended to decrease as lysine was added (Table 9).

3.3.2. Gene expression by treatment in PLAG1 GC type

The mRNA expression levels were significant for MyoG ($p = 0.0361$) and Myf5 ($p = 0.0206$), and there was a highly significant difference for Pax7 ($p < 0.0001$) and FASN ($p < 0.0001$). There was no significant difference in the expression level of MyoD between the control and treatment groups (Table 10).

3.3.3. Gene expression by treatment in PLAG1 CC type

There was a significant difference in MyoD ($p = 0.0014$) in PLAG1 CC type, with T3 having the highest relative gene expression level at 1.63 and T6 having the lowest at 0.73. The MyoG ($p < 0.0009$) had the lowest gene expression level in T6 at 0.28, which was significantly different from C2, T4, and T5. In MyoG, the addition of lysine had a negative effect in all treatments, with the amount of mRNA expression decreasing. There was no significant difference between the control and

Table 5
Gene expression level in PLAG1 GG type by glucose concentration and in the presence and absence of lysine in Hanwoo muscle satellite cells proliferation.

Gene	Genotype (GG)								SEM[c]
	High glucose[a]				Low glucose[b]				
	Lys –	Lys 2 mM	Lys 4 mM	Lys 8 mM	Lys –	Lys 2 mM	Lys 4 mM	Lys 8 mM	
MyoD	1.45	1.78	2.09	2.34	1.15	1.02	1.08	0.94	0.14
MyoG	1.17^{ab}	1.62^{a}	1.50^{a}	1.06^{ab}	1.13^{ab}	1.00^{ab}	0.89^{ab}	0.79^{b}	0.07
Myf5	1.17	1.42	1.61	1.84	1.31	1.53	1.46	2.13	0.09
Pax7	0.91^{bc}	1.24^{bc}	1.01^{bc}	1.13^{bc}	1.64^{bc}	8.32^{a}	4.40^{abc}	2.67^{b}	0.55
FASN	1.05	1.20	1.42	1.32	1.19	1.54	1.80	1.30	0.08

abc Values with different letters within the same column have significant differences ($p < 0.05$).
[a] C1, High glucose; T1, High glucose + Lys 2 mM; T2, High glucose + Lys 4 mM; T3, High glucose + Lys 8 mM.
[b] C2, Low glucose; T4, Low glucose + Lys2mM; T5, Low glucose + Lys 4 mM; T6, Low glucose + Lys 8 mM.
[c] Standard error of mean.

Table 6
Gene expression level in PLAG1 GC type by glucose concentration and in the presence and absence of lysine in Hanwoo muscle satellite cells proliferation.

Gene	Genotype (GC)								SEM[c]
	High glucose[a]				Low glucose[b]				
	Lys –	Lys 2 mM	Lys 4 mM	Lys 8 mM	Lys –	Lys 2 mM	Lys 4 mM	Lys 8 mM	
MyoD	1.61^{c}	3.54^{cb}	6.65^{b}	9.64^{b}	1.21^{c}	1.10^{c}	1.09^{c}	15.44^{a}	1.07
MyoG	0.45^{c}	0.94^{c}	4.34^{a}	2.79^{ac}	3.64^{a}	3.11^{a}	3.46^{a}	1.65^{abc}	0.30
Myf5	1.00^{ab}	1.03^{ab}	1.36^{ab}	1.67^{a}	1.15^{ab}	1.20^{ab}	0.58^{b}	1.43^{ab}	0.09
Pax7	1.15^{c}	0.94^{c}	1.58^{bc}	1.40^{bc}	4.25^{a}	2.85^{ab}	3.09^{a}	2.98^{ab}	0.25
FASN	0.97	0.98	1.55	0.92	1.71	1.55	1.12	1.21	0.09

abc Values with different letters within the same column have significant differences ($p < 0.05$).
[a] C1, High glucose; T1, High glucose + Lys 2 mM; T2, High glucose + Lys 4 mM; T3, High glucose + Lys 8 mM.
[b] C2, Low glucose; T4, Low glucose + Lys2mM; T5, Low glucose + Lys 4 mM; T6, Low glucose + Lys 8 mM.
[c] Standard error of mean.

Table 7
Gene expression level in PLAG1 CC type by glucose concentration and in the presence and absence of lysine in Hanwoo muscle satellite cells proliferation.

Gene	Genotype (CC)								SEM[c]
	High glucose[a]				Low glucose[b]				
	Lys –	Lys 2 mM	Lys 4 mM	Lys 8 mM	Lys –	Lys 2 mM	Lys 4 mM	Lys 8 mM	
MyoD	1.20^{c}	1.50^{c}	1.76^{c}	29.15^{b}	0.65^{c}	2.47^{c}	3.73^{c}	38.47^{a}	2.98
MyoG	0.75^{c}	0.50^{c}	0.23^{c}	19.83^{a}	0.58^{c}	2.64^{bc}	2.66^{bc}	5.85^{abc}	1.38
Myf5	1.23^{b}	1.42^{b}	1.76^{b}	1.60^{b}	2.71^{a}	1.15^{b}	1.15^{b}	1.12^{b}	0.12
Pax7	1.08	0.45	0.36	0.41	0.98	0.71	0.44	0.43	0.08
FASN	1.33^{bc}	1.27^{bc}	2.13^{abc}	1.00^{bc}	1.40^{bc}	0.77^{c}	2.63^{a}	3.00^{a}	0.18

abc Values with different letters within the same column have significant differences ($p < 0.05$).
[a] C1, High glucose; T1, High glucose + Lys 2 mM; T2, High glucose + Lys 4 mM; T3, High glucose + Lys 8 mM.
[b] C2, Low glucose; T4, Low glucose + Lys2mM; T5, Low glucose + Lys 4 mM; T6, Low glucose + Lys 8 mM.
[c] Standard error of mean.

Table 8
Effects of genotype, Glucose, and Lysine on gene expression in Hanwoo bovine muscle satellite cells proliferation.

Gene	Main effects							SEM[a]	Treatment p-values		
	Genotype			Glucose		Lysine					
	GG	GC	CC	LG[b]	HG[c]	–	2,4,8 mM		Genotype	Glucose	Lysine
MyoD	1.48^{b}	5.04^{ab}	9.87^{a}	5.70	5.23	1.21	6.88	1.14	0.007	0.835	<0.001
MyoG	1.14^{b}	2.55^{ab}	4.13^{a}	2.28	2.93	1.29	3.05	0.39	0.041	0.509	<0.005
Myf5	1.56^{a}	1.18^{b}	1.52^{a}	1.41	1.43	1.43	1.40	0.05	0.016	0.902	0.488
Pax7	2.66^{a}	2.28^{a}	0.61^{b}	2.73	0.97	1.67	1.91	0.31	<0.001	<0.001	0.080
FASN	1.35^{ab}	1.25^{b}	1.69^{a}	1.60	1.26	1.27	1.48	0.07	0.038	0.021	0.177

ab) Values with different letters within the same column have significant differences ($p < 0.05$).
[a] Standard error of mean.
[b] Low glucose.
[c] High glucose.

Table 9
Gene expression level in PLAG1 GG type by glucose concentration and in the presence and absence of lysine in Hanwoo muscle satellite cells differentiation.

Gene	Genotype (GG)								SEM[c]
	High glucose[a]				Low glucose[b]				
	Lys –	Lys 2 mM	Lys 4 mM	Lys 8 mM	Lys –	Lys 2 mM	Lys 4 mM	Lys 8 mM	
MyoD	1.28	2.18	1.81	1.35	1.21	1.25	1.52	0.78	0.16
MyoG	1.95^{ab}	1.91^{ab}	1.77^{b}	1.15^{ab}	3.06^{a}	1.34^{ab}	1.47^{ab}	0.53^{b}	0.18
Myf5	1.30	1.19	1.34	1.51	1.20	1.04	1.60	1.55	0.06
Pax7	1.14^{b}	10.79^{b}	8.75^{b}	18.13^{b}	5.70^{b}	5.77^{b}	46.45^{a}	54.88^{a}	4.20
FASN	1.06^{a}	0.95^{ab}	0.72^{ab}	1.16^{a}	0.81^{ab}	0.53^{ab}	0.52^{ab}	0.27^{b}	0.07

ab Values with different letters within the same column have significant differences ($p < 0.05$).
[a] C1, High glucose; T1, High glucose + Lys 2 mM; T2, High glucose + Lys 4 mM; T3, High glucose + Lys 8 mM.
[b] C2, Low glucose; T4, Low glucose + Lys2mM; T5, Low glucose + Lys 4 mM; T6, Low glucose + Lys 8 mM.
[c] Standard error of mean.

Table 10
Gene expression level in PLAG1 GC type by glucose concentration and in the presence and absence of lysine in Hanwoo muscle satellite cells differentiation.

Gene	Genotype (GC)								SEM[c]
	High glucose[a]				Low glucose[b]				
	Lys –	Lys 2 mM	Lys 4 mM	Lys 8 mM	Lys –	Lys 2 mM	Lys 4 mM	Lys 8 mM	
MyoD	1.10	0.96	1.03	0.58	0.93	0.87	1.07	0.39	0.07
MyoG	1.00^{a}	0.60^{ab}	0.66^{ab}	0.31^{b}	0.51^{ab}	0.41^{ab}	0.41^{ab}	0.29^{b}	0.06
Myf5	0.98^{b}	2.10^{ab}	2.56^{a}	2.56^{a}	1.86^{ab}	1.97^{ab}	2.42^{ab}	2.68^{a}	0.14
Pax7	0.86^{b}	2.66^{a}	3.56^{a}	3.06^{a}	3.13^{a}	3.05^{a}	3.02^{a}	0.81^{b}	0.23
FASN	1.06^{b}	0.60^{b}	0.68^{b}	2.71^{a}	0.52^{b}	0.52^{b}	2.40^{a}	1.62^{ab}	0.19

ab Values with different letters within the same column have significant differences ($p < 0.05$).
[a] C1, High glucose; T1, High glucose + Lys 2 mM; T2, High glucose + Lys 4 mM; T3, High glucose + Lys 8 mM.
[b] C2, Low glucose; T4, Low glucose + Lys2mM; T5, Low glucose + Lys 4 mM; T6, Low glucose + Lys 8 mM.
[c] Standard error of mean.

treatment groups for Myf5 ($p = 0.2297$), and it was very significant for Pax7 ($p < 0.0001$) and FASN ($p = 0.001$) (Table 11).

3.3.4. Main effects of genotype, glucose, and lysine on differentiation

The genotype (GG, GC, CC) major effects in differentiation showed significant differences in gene expression of MyoD ($p = 0.0030$), MyoG ($p < 0.0001$), Myf5 ($p < 0.0001$), Pax7 ($p < 0.0001$), and FASN ($p = 0.0018$). For MyoD, MyoG, and Pax7, gene expression levels of the PLAG1 GG type were highest. Among them, the relative gene expression level of Pax7 in the GG type was 18.95, which was about 6 times higher than that in the GC type. In Myf5 and FASN, the PLAG1 GC type had a high mRNA expression level. The main effect of glucose was MyoD ($p = 0.1679$), MyoG ($p = 0.6481$), Myf5 ($p = 0.4791$), Pax7 ($p = 0.0671$), and FASN ($p = 0.5966$), with no significant differences in all genes. Lysine treatment affected MyoG ($p < 0.0001$), Myf5 ($p = 0.0005$), and Pax7 ($p = 0.0028$). Among these, MyoG showed that the gene expression level decreased as lysine was treated. There was no significant difference in Myf5 ($p = 0.6767$) and FASN ($p = 0.9436$) (Table 12).

3.4. Cell viability by genotype (PLAG1 GG, GC, CC)

There was no significant difference in cell viability between PLAG1 CC and GC types, but there was a significant difference in GG type ($p = 0.0011$). In PLAG1 GG type, survival was higher in C1 and C2 without lysine, and C1 was significantly different from T5 and T6. Additionally, C2 was significantly different from T6, and T1 was significantly different from T6. This result showed that the viability of cells tended to decrease as lysine level was increased (Fig. 2).

3.5. Cell differentiation by control and treatment medium

Based on the different medium compositions (C1~T6), bovine

Table 11
Gene expression level in PLAG1 CC type by glucose con and in the presence and absence of lysine in Hanwoo muscle satellite cells differentiation.

Gene	Genotype (CC)								SEM[c]
	High glucose[a]				Low glucose[b]				
	Lys –	Lys 2 mM	Lys 4 mM	Lys 8 mM	Lys –	Lys 2 mM	Lys 4 mM	Lys 8 mM	
MyoD	0.94^{cd}	0.91^{cd}	1.12^{abc}	1.63^{a}	1.22^{abc}	1.22^{abc}	1.42^{abc}	0.73^{d}	0.07
MyoG	0.93^{ab}	0.59^{bc}	0.80^{bc}	0.44^{bc}	1.20^{a}	0.75^{ab}	0.90^{ab}	0.28^{c}	0.07
Myf5	0.82	1.01	1.01	1.36	1.24	1.06	1.33	1.08	0.06
Pax7	1.12^{b}	1.19^{b}	1.82^{b}	8.94^{a}	7.11^{a}	1.02^{b}	2.77^{ab}	1.33^{b}	0.67
FASN	0.62^{b}	0.58^{b}	0.46^{b}	0.63^{b}	1.27^{a}	0.83^{ab}	0.51^{b}	0.46^{b}	0.06

abcd Values with different letters within the same column have significant differences ($p < 0.05$).
[a] C1, High glucose; T1, High glucose + Lys 2 mM; T2, High glucose + Lys 4 mM; T3, High glucose + Lys 8 mM.
[b] C2, Low glucose; T4, Low glucose + Lys2mM; T5, Low glucose + Lys 4 mM; T6, Low glucose + Lys 8 mM.
[c] Standard error of mean.

Table 12
Effects of Genotype, Glucose, and Lysine on gene expression in Hanwoo muscle satellite cells differentiation.

Gene	Main effects										
	Genotype			Glucose		Lysine		SEM[a]	Treatment P-values		
	GG	GC	CC	LG[b]	HG[c]	–	2,4,8 mM		Genotype	Glucose	Lysine
MyoD	1.42[a]	0.87[b]	1.15[ab]	1.24	1.05	1.11	1.16	0.06	0.0030	0.1679	0.6767
MyoG	1.65[a]	0.52[b]	0.74[b]	1.01	0.93	1.44	0.81	0.15	<0.0001	0.6481	<0.0001
Myf5	1.31[b]	2.19[a]	1.12[b]	1.48	1.59	1.23	1.63	0.13	<0.0001	0.4791	0.0005
Pax7	18.95[a]	2.52[b]	3.16[b]	5.17	11.25	3.18	9.89	2.28	<0.0001	0.0671	0.0028
FASN	0.75[b]	1.26[a]	0.67[b]	0.94	0.86	0.89	0.90	0.07	0.0018	0.5966	0.9436

ab) Values with different letters within the same column have significant differences ($p < 0.05$).
[a] Standard error of mean.
[b] Low glucose.
[c] High glucose.

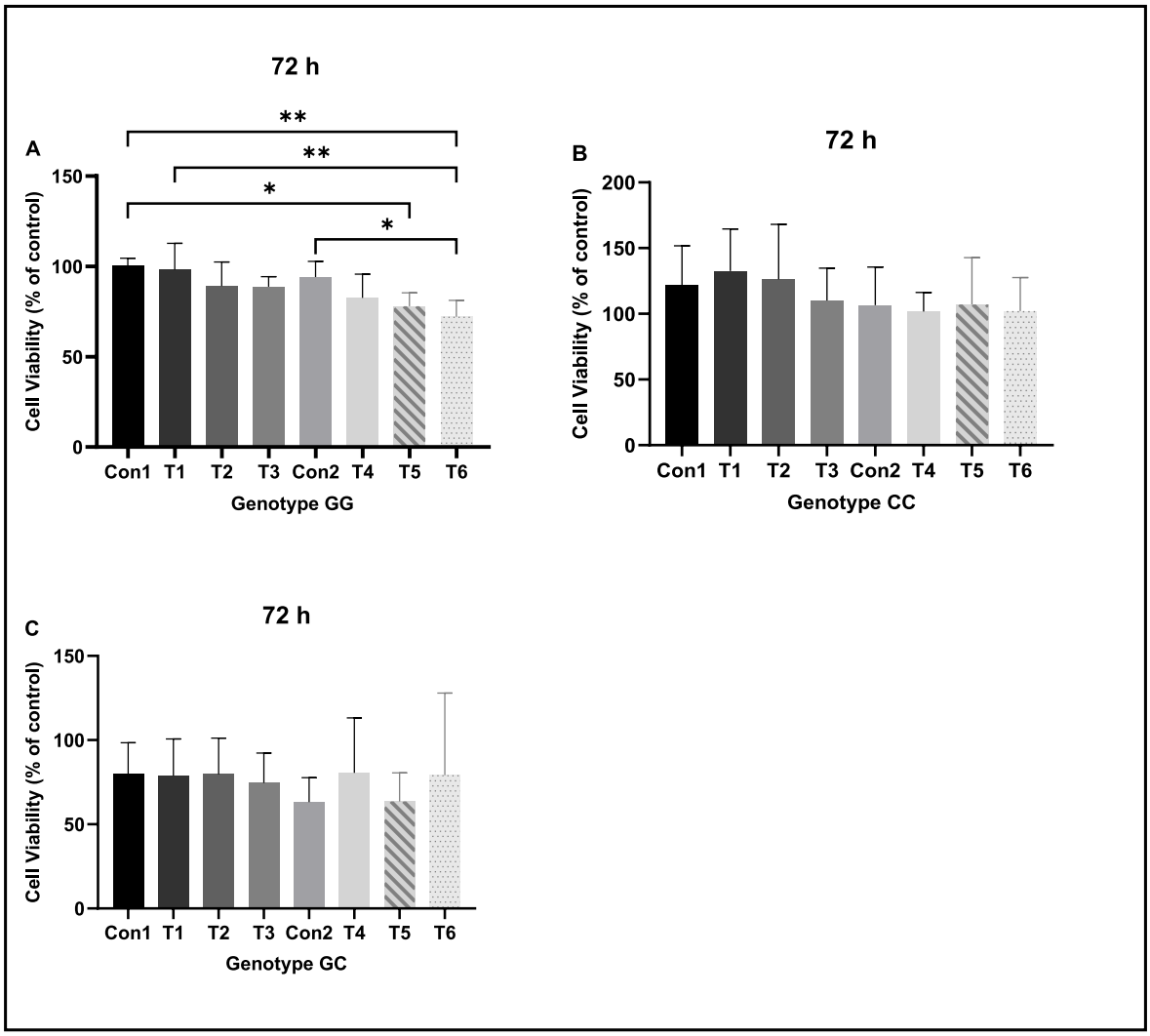

Fig. 2. Cell viability by genotype (PLAG1 GG, GC, CC).

muscle satellite cells were stained with Giemsa staining, MitoTracker and Hoechst 33,342 after 96 h of cell differentiation medium treatment (Fig. 3). The results of Giemsa staining revealed the formation of myotubes in all medium treatment groups. Particularly, cells treated with high glucose exhibited the formation of wider and thicker myotubes. To confirm this results, cells were also stained with Mitotracker along with Hoechst 3334, which showed similar results to Giemsa staining. However, it was observed that cell differentiation occurred slightly more when lysine was administered in lower amounts.

4. Discussion

PLAG1 has been reported to affect the growth of cattle by regulating development of muscle and fat (Wang et al., 2022b). This study aimed to enhance the production efficiency of cell-based food by selecting appropriate PLAG1 genotypes and determining the optimal concentration of glucose and lysine, energy and protein sources, to promote the growth and differentiation of muscle satellite cells. In our study, genotype analysis showed no significant differences among the GG, GC, and

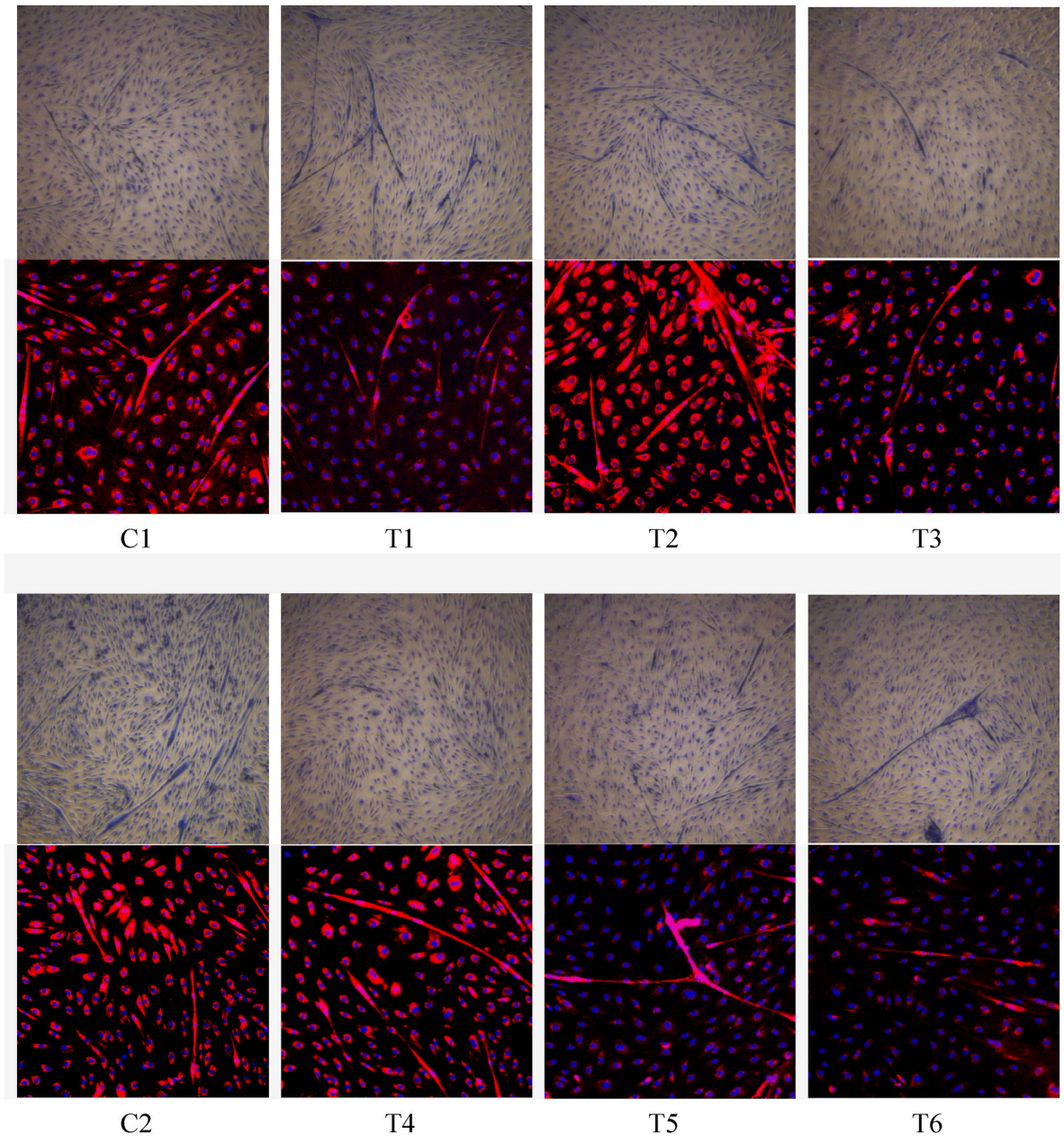

Fig. 3. Morphology of bovine muscle satellite cells with Giemsa, MicoTracker, and Hoechst 33,342 after 96 h differentiation, C1: High glucose; T1: High glucose + Lys 2 mM; T2: High glucose + Lys 4 mM; T3: High glucose + Lys 8 mM; C2: Low glucose; T4: Low glucose + Lys2mM; T5: Low glucose + Lys 4 mM; T6: Low glucose + Lys 8 mM, Scale bar = 1000 μm.

CC genotypes, although the GG genotype exhibited numerically higher results. The variations in phenotypic traits such as dressed weight, yield grade, and marbling score emphasize the potential influence of genotype on certain physical characteristics. This aligns with previous studies that have highlighted the association between PLAG1 and bovine conformation traits (Karim et al., 2011; Utsunomiya et al., 2017; Littlejohn et al., 2012). Specifically, in Japanese black cattle, specific loci like PLAG1 QTL BTA14 and BTA6 have shown correlations with carcass weight, indicating the relevance of genetic factors in determining physical attributes (Zhao et al., 2012). Regarding the gene expression levels in PLAG1 GG type, our findings revealed that gene expression tended to fluctuate based on lysine supplementation levels in different glucose conditions, suggesting a complex interplay between glucose availability and lysine supplementation in gene regulation. Conversely, in the GC type, lysine supplementation led to consistent increases in gene expression across treatments, indicating a more straightforward response to lysine supplementation. Concomitant to our results, previous studies demonstrated that cell proliferation is more active under low glucose, but cell differentiation with related expression levels of mRNA and protein in the presence of high glucose medium (Furuichi et al., 2021; Yue et al., 2010). This nuanced understanding of gene expression dynamics underscores the importance of considering genotype-specific responses in cellular studies, which mirrors previous research demonstrating genotype-specific responses in various biological processes.

Furthermore, the main effects of genotype, glucose, and lysine on proliferation elucidated significant differences in gene expression levels. The higher expression levels of MyoD, MyoG, and Pax7 genes in the GG type and higher expression levels of Myf5 and FASN genes in the GC type highlight the genotype-dependent regulation of proliferation-related genes. This aligns with previous studies suggesting that different genotypes may exhibit distinct molecular profiles and responses to external stimuli. The interactions between genotype, glucose, and lysine for

proliferation and differentiation provided additional insights into the complex regulatory mechanisms underlying cellular processes. While some interactions showed significance, others did not, indicating the multifaceted nature of gene regulation and the need for further investigation into the underlying mechanisms. This resonates with previous research demonstrating the intricate interplay between genetic, environmental, and nutritional factors in cellular processes. Moreover, the analysis of cell viability by genotype underscored the impact of genetic factors on cell survival rates, with the GG type showing higher viability in certain conditions. This underscores the importance of considering genotype-specific responses in cell culture experiments and highlights the potential implications for cell-based food production. Previous studies have also highlighted the influence of genetic factors on cell viability and growth potential, further emphasizing the relevance of our findings. Finally, the cell differentiation studies provided valuable insights into the effects of different medium compositions on myotube formation. The observation that cells treated with high glucose exhibited wider and thicker myotubes suggests that glucose availability plays a crucial role in promoting cell differentiation. This aligns with previous research indicating that glucose serves as a vital element for cell proliferation and differentiation, with low glucose conditions promoting cell proliferation and high glucose conditions enhancing cell differentiation. Previous study also demonstrated that rumen-protected lysine (RPLys) and rumen-protected methionine (RPMet) in low-protein feed increased daily weight gain in Holstein cattle (Zou et al., 2023).

In the study, the PLAG1 CC type showed higher relative gene expression than the GC and CC type in MyoD ($p < 0.001$), MyoG ($p < 0.001$), Myf5 ($p = 0.0038$), and FASN ($p = 0.0002$), of which the mRNA expression level was higher in the treatment group to which lysine 8 mM was added. Pax7 showed the highest gene expression level of PLAG1 CC type. In contrast, PLAG1 CC type tended to decrease mRNA expression level as lysine was added in Pax7. Due to the addition of lysine, the proliferation of cells increased to the more active tendencies 12.54 and 33.81. In addition, the PLAG1 CC type showed MyoG in the treatment group to which lysine 8 mM was added, and among them, when lysine 8 mM was added in the PLAG1 GC and CC type, the relative gene expression level of MyoD was higher, respectively. For MyoD, the gene expression level of the treatment group to which lysine 8 mM was added to high glucose was 13.71, and the gene expression level of the treatment group to which lysine 8 mM was added to low glucose was 18.28. This showed that cell proliferation was more activated when the low glucose medium was used as in previous research results (Furuichi et al., 2021). The PLAG1 GG-type and low glucose-type treatment group had a gene expression level of 4.26, which was about six times higher than that of the PLAG1 CC type. In terms of cell differentiation, the PLAG1 GC type's gene expression level was high in MyoD, Myf5, and FASN, and in MyoG and Pax7, the PLAG1 GG type's gene expression level was high. MyoG had the highest expression level of C2 treated with medium added with only high glucose at 3.06, and Pax7 had high mRNA expression levels of 46.45 and 54.88 in T5 and T6 treated with lysine in low glucose, respectively. In the PLAG1 GG type, as lysine was added, the gene expression level of Pax7 continued to increase to Control 3.42, lysine 2 mM treatment group 8.28, Lysine 4 mM treatment group 27.60, and lysine 8 mM treatment group 36.50 as the lysine was added. In addition, Pax7 showed higher gene expression levels in the treatment group using low glucose medium than in the treatment group using high glucose medium. Therefore, in the differentiation of cells, Pax7 tended to have higher mRNA expression levels in the PLAG1 GG type, the treatment group with low glucose, and the treatment group with lysine. These results show that the addition of lysine and glucose to Korean cattle muscle satellite cells can promote protein biosynthesis, thereby enhancing cell proliferation and myotomy.

5. Conclusion

In conclusion, our study provides valuable insights into the genotype variations of the PLAG1 genotype in Hanwoo skeletal muscle satellite cells in response to different levels of glucose and lysine. In the analysis of Hanwoo carcass traits, the PLAG1 GG genotype exhibited numerically greater yield grade and marbling scores. Additionally, Hanwoo muscle satellite cells isolated form PLAG1 GG type showed higher survival rate. The cell differentiation results indicated that the high glucose and low lysine treatment group formed wider and thicker myotubes. Genes related to growth and development of muscle and fat were dynamically regulated by PLAG1 genotype as well as glucose and lysine treatments. Results from this study provides useful information about growth and development of Hanwoo muscle satellite cells classified by different PLAG genotype. By treating these cells with essential nutrients like glucose and lysine, we provide critical information for selecting optimal cells as a primary source for cell-based food production. Future research into the molecular mechanisms underlying these processes will be warranted for maximizing the production efficiency of cell-based food.

CRediT authorship contribution statement

Hyojin Kim: Conceptualization, Formal analysis, Writing – review & editing. **Sungkwon Park:** Formal analysis, Methodology, Software, Writing – original draft, and. **Bosung Kim:** Formal analysis, Methodology, Software, Writing – original draft. **Minji Kim:** Validation, Writing – review & editing. **Tae Hyung Lee:** Validation, Writing – review & editing, and. **Jia Yu:** Validation, Writing – review & editing. **Il Soo Park:** Conceptualization, Writing – original draft. **Sun Jin Hur:** Conceptualization, Writing – original draft. **Xiang Zi Li:** Validation, Writing – review & editing, and. **Seong Ho Choi:** Validation, Writing – review & editing.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100879.

Data availability

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

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