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Transcriptome changes associated with fat deposition in the *longissimus thoracis* of Korean cattle following castration

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Abstract

The castration of bulls increases the intramuscular fat (IMF) content in skeletal muscle. However, the biological processes of IMF accumulation in skeletal muscle after castration are not completely understood at the molecular level. This study examined the global transcriptomic changes in the *longissimus thoracis* muscle (LT) of bulls following castration using RNA sequencing (RNA-Seq) and identified new genes or pathways associated with beef quality. Ten bulls and 10 steers castrated at 6 months of age were slaughtered at 26 and 32 months of age respectively. For transcriptome analysis, six LT samples from three bulls and three steers were selected based on age, carcass weight, carcass quantity and beef quality grades. Using RNA-Seq, transcriptomic profiles of the LT were compared between bulls and steers. In all, 640 of the 18,027 genes identified through RNA-Seq were differentially expressed genes (DEGs) between bulls and steers. Pathway analysis of these 640 DEGs showed significant ($p < .05$) changes in seven Kyoto Encyclopedia of Genes and Genomes pathways, and the most significant terms were complement and coagulation cascade pathways. The transcriptomic expression patterns of 10 genes in the complement and coagulation cascades were validated using all animals through quantitative real-time polymerase chain reaction analysis. In conclusion, transcriptome changes associated with the complement and coagulation cascade pathways provide novel insights into understanding molecular mechanisms responsible for IMF accumulation following castration in beef cattle.

KEYWORDS
castration, intramuscular fat, Korean cattle, *Longissimus thoracis* muscle, transcriptome

1 | INTRODUCTION

Castration is a reliable method of increasing the intramuscular fat (IMF) content (marbling) of the *longissimus thoracis* (LT) muscle (Bong et al., 2012; Jacobs et al., 1977; Park et al., 2002). Increased marbling positively affects beef tenderness, juiciness, flavour, and overall palatability and acceptability (Baik et al., 2017; Hausman et al., 2009; Park, Beak, et al., 2018). Previously, we examined the molecular mechanisms responsible for the increased IMF deposition following castration of Korean cattle. One study revealed that castration altered expression of many lipid metabolism genes in the LT, including those involved in lipid uptake, lipogenesis, insulin signalling and lipolysis (Bong et al., 2012). A microarray study revealed that castration altered the expression of genes involved in adipogenesis, fatty acid oxidation, oxidative phosphorylation and the tricarboxylic acid cycle in the *longissimus dorsi* muscle (Jeong et al., 2013).

In laboratory animals and human studies, changes in immune factors and the tissue complement pathway were associated with lipid metabolism and obesity (Marti, Marcos, & Martinez, 2001; Pugia, 2015; Schaffler & Scholmerich, 2010). The transcriptomes of IMF tissues

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between Chinese Qinchuan bulls and steers were compared using RNA-Seq (Zhang et al., 2017). That study revealed that castration up-regulated genes related to lipogenesis and fatty acid signalling pathways and found gene expression changes in immune factors and intracellular signal transduction pathways following castration. However, no report has examined the association of LT tissue immune factors and complement pathway with the increased IMF deposition following castration.

RNA sequencing (RNA-Seq) is a high-throughput sequencing method that can be used to identify and quantify transcripts. By contrast with microarray analysis, RNA-Seq can detect all transcripts, including those of unexpected and unidentified genes (Wang, Gerstein, & Snyder, 2009). Transcriptomic changes in the muscle tissue of cattle following castration have not yet been examined with RNA-Seq. In this study, we performed RNA-Seq analysis to examine the transcriptomic changes associated with beef quality in the LT of Korean cattle following castration. Transcriptomic profiles of bulls and steers in the LT were compared, and qPCR analysis was conducted to confirm the RNA-Seq results.

2 | MATERIALS AND METHODS

2.1 | Animals and tissue sampling

All experimental procedures were performed in accordance with the Animal Experimental Guidelines provided by the Seoul National University Institutional Animal Use and Care Committee, Republic of Korea. The experimental protocol was approved by the Seoul National University Institutional Animal Use and Care Committee (SNU-151221–4).

Ten Korean cattle bulls and 10 Korean cattle steers were used in this study. Steers were castrated at 6 months of age, and animals were reared under the same feeding conditions, as described in a previous study (Bong et al., 2012). We have used bulls and steers as an experimental model of clear differences in marbling to understand transcriptome changes relevant to IMF accumulation following castration. We castrated bulls under the guidance of an expert veterinarian. Bulls and steers were slaughtered at mean ages of 26 and 32 months, respectively, and their carcass weights were 445.2 ± 9.37 kg and 434.3 ± 9.47 kg. The cattle were slaughtered in a conventional manner after captive bolt stunning and exsanguination. Immediately after slaughter, pieces of the LT tissue between the 12th and the 13th ribs were taken from the hot carcass, frozen in liquid nitrogen and stored frozen at −70°C until analysis. Carcass weight and beef grade, including back-fat thickness, rib eye area, yield index, yield grade, marbling score (MS) and quality grade (QG), were evaluated using the method described in our previous study (Piao et al., 2017). The IMF contents were measured as described by Folch, Lees, and Stanley (1957). We separated the muscle and IMF portions from the LM of the steers, as described previously (Bong et al., 2012). Briefly, we manually excised IMF from the frozen LT of steers using tweezers and scissors. To minimize the degradation of RNA in the muscle and fat tissues, the tissues were placed on dry ice. It was impossible to separate the IMF from the LT of bulls because the IMF was rarely visible in the bulls due to minimal deposition.

2.2 | RNA extraction, library preparation and sequencing

Total RNA was isolated from LT tissue using TRIzol reagent (Molecular Research Center) according to the manufacturer's protocol. Total RNA was quantified by monitoring the absorbance at 260 nm, and total RNA integrity was checked using agarose gel electrophoresis and ethidium bromide staining of the 28S and 18S bands. Ribonucleic acid quality was also checked using the RNA 6000 Nano LabChip kit and Agilent 2100 Bioanalyzer (Agilent Technologies). RNA samples were stored at −70°C until RNA-Seq and qPCR analyses.

For high-throughput sequencing, we used RNA-Seq samples from three bulls and three steers. We selected the animals for RNA-Seq that had values similar to the average for all animals (10/group) in terms of carcass quantity, meat quality and carcass weight (Table 1). cDNA library construction and sequencing analysis were conducted at the C&K Genomics Bioinformatics (C&K Genomics). Briefly, selected total RNA samples were re-quantified through a quantitative immunofluorescence assay using the Quant-iT RiboGreen RNA Assay Kit (Invitrogen). The mRNA library was prepared using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina), and its quality was checked with the Agilent DNA 1000 Kit (Agilent Technologies). The library was sequenced based on the Illumina NextSeq 500 protocol to create 76-bp length paired-end reads. The quality of the sequencing reads from LT tissues was checked using FastQC. The reads that passed quality control were mapped to the *Bos taurus* genome (UMD3.1) from UCSC using Tophat2 (v2.0.2) and counted using HTSeq (v0.5.3p3).

2.3 | Identification and annotation of differentially expressed genes

To identify differentially expressed genes (DEGs), we processed the raw RNA-Seq data of each sample with the trimmed mean of *M*-values normalization method using edgeR (Robinson, McCarthy, & Smyth, 2010). We selected significant DEGs at FDR < 0.05 and $|\log_2 FC| \geq 1.5$ for functional annotation of DEGs. The DEGs were categorized in terms of gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool (v6.7) (Dennis et al., 2003).

2.4 | Quantitative real-time PCR

All total RNA extracts from LT tissues were used for qPCR. cDNA was synthesized from the RNA templates (2 μg) using the iScript cDNA

TABLE 1 Carcass characteristics of Korean bulls and steers of all animal and animals for RNA sequencing

Variables	All animals				Animals for RNA sequencing			
	Bulls (n = 10)	Steers (n = 10)	*SEM*	*p*-value	Bulls (n = 3)	Steers (n = 3)	*SEM*	*p*-value
Carcass weight, kg	445	434	6.60	.42	432	435	10.7	.93
Back-fat thickness, mm	4.70	11.7	0.94	<.001	6.33	12.7	1.65	.07
Rib eye area, cm	86.4	95.9	3.81	.23	84.3	99.0	8.52	.46
Yield index[a]	69.0	66.6	0.57	.04	68.0	65.9	0.89	.33
Yield grade[b]	30.0	22.0	1.34	.003	30.0	23.3	2.11	.18
Intramuscular fat (%)	4.18	16.7	1.71	<.001	6.20	18.8	3.16	.05
Marbling score[c]	1.10	7.10	0.71	<.001	1.33	6.67	1.24	<.01
Quality grade[d]	11.0	44.0	3.90	<.001	13.3	43.3	7.03	<.01

[a]Yield index: 68.184 − 0.625 × back-fat thickness + 0.13 × rib eye area − 0.024 × Carcass weight + 3.23.

[b]Yield grade: C (yield index < 63.3) = 10; B (63.3 ≤ yield index < 67.2) = 20; A (yield index ≥ 67.2) = 30.

[c]Marbling score: 1 = min; 9 = max.

[d]Quality grade: 50 = 1++; 40 = 1+; 30 = 1; 20 = 2; 10 = 3.

synthesis kit (Bio-Rad). Quantitative real-time PCR was performed as reported elsewhere (Fassah, Jeong, & Baik, 2018; Park, Kang, Na, Lee, & Baik, 2018) using QuantiTect SYBR Green RT-PCR Master Mix (Qiagen). Quantitative real-time PCR analyses were performed using Rotor-Gene Q (Qiagen) in a 25 µl total reaction volume. The mixture contained 20 ng cDNA, 1.25 µl of 10 µM primers and 12.5 µl SYBR Green RT-PCR Master Mix. The reaction was initiated with denaturation (95°C for 15 min), followed by 40 cycles consisting of denaturation (94°C for 15 s), annealing (55°C for 30 s) and elongation (72°C for 30 s) steps. Primer information is presented in Table S1. The ΔΔCT method was used to determine relative fold changes (Livak & Schmittgen, 2001). Quantitative real-time PCR data of LT for the comparison between bulls and steers were normalized relative to the housekeeping gene *β-actin*, and qPCR data comparing IMF and muscle tissues were normalized to the reference gene ribosomal protein lateral stalk subunit P0, as validated in our previous study (Park, Kang, et al., 2018).

2.5 | Statistical analysis

All data are expressed as the mean + *SE*. Statistical differences between bulls and steers were determined using the general linear model procedure in SAS 9.4 software (SAS Institute).

3 | RESULTS

3.1 | Carcass characteristics of bulls and steers

We compared the carcass characteristics between Korean cattle bulls and steers to confirm the effects of castration on carcass traits (Table 1). The bulls had higher yield indices (p = .04) and yield grades (p = .003). Meanwhile, the steers had deeper back-fat thickness (p < .0001), higher MS (p < .0001) and IMF content (p < .001), and better QG (p < .0001). Similarly, the steers selected for RNA-Seq had higher MS (p < .01) and IMF content (p = .05) and better QG (p < .01; Table 1). These results, except for those for the rib eye area, corresponded to those of our previous study (Bong et al., 2012), revealing increased beef quality following the castration of bulls.

3.2 | RNA-Seq results and DEG identification

In total, six cDNA libraries were constructed, and an average of 57,928,004 paired-end 76-bp sequence reads was generated. At least 87.0% of reads in each sample were mapped to the bovine reference genome (UMD3.1) using Tophat2 (v2.0.2) (Table S2).

EdgeR was used to identify DEGs between bulls and steers in LT tissues. In total, 640 DEGs were identified (FDR < 0.05 and $|\log_2 FC| \geq 1.5$), with 333 up-regulated genes and 307 down-regulated genes in steers. The up-regulated and down-regulated genes included 296 and 272 protein-coding genes, 8 and 6 miRNAs, 5 and 2 pseudogenes, and 24 and 27 uncharacterized genes respectively (Tables S3 and S4). In this study, very few miRNAs might have been detected because we used total RNAs without miRNA enrichment.

3.3 | Functional annotation

We used the DAVID tool (v6.7) for functional annotation of 640 DEGs. The DEGs were categorized on based on three GO categories

(biological process [BP], molecular function [MF] and cellular component [CC]) and KEGG pathways (Tables S5–S7). In the BP pathway, immune system-related GO terms were most significantly enriched, including the wound response (p = 2.76E-12), acute inflammatory response (p = 6.64E-10) and inflammatory response (p = 1.01E-09; Table S5). In the MF pathway, enzyme inhibitor-related GO terms were most significantly enriched, including enzyme inhibitor activity (p = 1.37E-08), endopeptidase inhibitor activity (p = 5.75E-07), peptidase inhibitor activity (p = 2.07E-06) and serine-type endopeptidase inhibitor activity (p = 9.10E-06; Table S6). Finally, the most significant GO terms in the CC pathway were related to the extracellular region, including the terms extracellular region (p = 1.15E-18), extracellular space (p = 4.29E-15) and extracellular region part (p = 1.89E-11; Table S7).

According to the KEGG pathway results, the DEGs largely clustered into seven representative terms, including the complement and coagulation cascades, peroxisome proliferator-activated receptor (PPAR) signalling pathway, retinol metabolism, metabolism of xenobiotics by cytochrome P450, drug metabolism by cytochrome P450, drug metabolism by other enzymes and tyrosine metabolism (Table 2). The two most significant terms were complement and coagulation cascades and the PPAR signalling pathway. Figure 1 shows the networks of DEGs involved in the complement and coagulation cascades and the PPAR signalling pathway.

Twenty-two DEGs were involved in the complement and coagulation cascade pathways (Table 2). Among these, five genes were up-regulated in steers, including *coagulation factor 3, tissue factor* (*F3*), *serpin family E member 1* (*SERPINE1*), *plasminogen activator, urokinase receptor* (*PLAUR*), *complement C5a receptor 1* (*C5AR1*) and *complement C4A* (*C4A*). The other 17 genes, including *coagulation factor 2* (*F2*), *coagulation factor 10* (*F10*), *kininogen 1* (*KNG1*), *plasminogen* (*PLG*), *serpin family A member 1* (*SERPINA1*), *serpin family C member 1* (*SERPINC1*), *serpin family D member 1* (*SERPIND1*), *serpin family F member 2* (*SERPINF2*), *carboxypeptidase B2* (*CPB2*), *protein C, inactivator of coagulation factors Va and VIIIa* (*PROC*), *fibrinogen alpha chain* (*FGA*), *fibrinogen gamma chain* (*FGG*), *complement component 4 binding protein alpha* (*C4BPA*), *complement C5* (*C5*), *complement C8 alpha chain* (*C8A*), *complement C9* (*C9*), and *mannan-binding lectin serine peptidase 1* (*MASP1*), were down-regulated in steers.

Fourteen DEGs were involved in the PPAR signalling pathway (Table 2). Six genes, including *peroxisome proliferator-activated receptor gamma* (*PPARG*), *fatty acid-binding protein 4* (*FABP4*), *perilipin 1* (*PLIN1*), *adiponectin, C1Q and collagen domain containing* (*ADIPOQ*), *acyl-CoA synthetase long chain family member 5* (*ACSL5*), and *phosphoenolpyruvate carboxykinase 2, mitochondrial* (*PCK2*), were up-regulated in steers. The remaining eight DEGs, including *fatty acid-binding protein 1* (*FABP1*), *solute carrier family 27 member 1* (*SLC27A1*), *solute carrier family 27 member 5* (*SLC27A5*), *solute carrier family 27 member 6* (*SLC27A6*), *apolipoprotein A2* (*APOA2*), *apolipoprotein C3* (*APOC3*), *fatty acid desaturase 2* (*FADS2*), and *cytochrome P450 family 4 subfamily A polypeptide 11* (*CYP4A11*), were down-regulated in steers.

3.4 | Validation of DEGs through qPCR

The complement and coagulation pathway was the most enriched category among KEGG terms in our analysis. There were 22 DEGs involved in complement and coagulation pathways (Table 2). Of the 22 DEGs, we selected 10 genes that exhibited large differences between bulls and steers, and we performed qPCR to validate the DEGs (Table 3). The results of quantita

tive real-time PCR analysis were consistent with those of RNA-Seq analysis for all genes tested, although the data for some genes did not reach statistical significance. Among the five up-regulated genes, the mRNA levels of four genes (*F3*, *SERPINE1*, *PLAUR* and *C5AR1*) were higher (p < .05) in steers than in bulls. The mRNA levels of *MASP1* were lower (p < .05) in steers compared to bulls.

3.5 | Comparison of mRNA expression levels between muscle and IMF through qPCR

We compared the mRNA expression levels of the muscle and IMF portions of the LT from steers. Of the 12 selected genes, expression

TABLE 2 KEGG pathway terms for DEGs between bulls and steers in the *longissimus thoracis* muscle

Order	KEGG pathway term	*p*-value	Count	Genes
1	Complement and coagulation cascades	1.00E−13	22	*F2, F3, F10, PLG, PLAUR, MASP1, FGA, FGG, SERPINA1, SERPINC1, SERPIND1, SERPINE1, SERPINF2, KNG1, CPB2, PROC, C4A, C4BPA, C5, C5AR1, C8A, C9*
2	PPAR signalling pathway	1.39E−06	14	*FABP1, FABP4, PLIN1, CYP4A11, SLC27A1, SLC27A5, SLC27A6, PCK2, PPARG, APOA2, APOC3, ADIPOQ, ACSL5, FADS2*
3	Retinol metabolism	2.67E−05	10	*CYP1A2, CYP2B6, CYP3A5, CYP4A11, ADH4, ADH6, DGAT2, RDH16,* ENSBTAG00000040337, ENSBTAG00000034189
4	Metabolism of xenobiotics by cytochrome P450	1.83E−04	9	*CYP1A3, CYP2B6, CYP2E1, CYP3A5, ADH4, ADH6, MGST1,* ENSBTAG00000040337, ENSBTAG00000034189
5	Drug metabolism—cytochrome P450	5.52E−04	9	*CYP1A3, CYP2B6, CYP2E1, CYP3A5, ADH4, ADH6, MGST1,* ENSBTAG00000040337, ENSBTAG00000034189
6	Drug metabolism—other enzymes	.009	6	*CYP3A5, CDA, UPB1, DPYS, UCK2, ENSBTAG00000040337*
7	Tyrosine metabolism	.009	6	*ADH4, ADH6, HPD, PNMT, SAO, ENSBTAG00000034189*

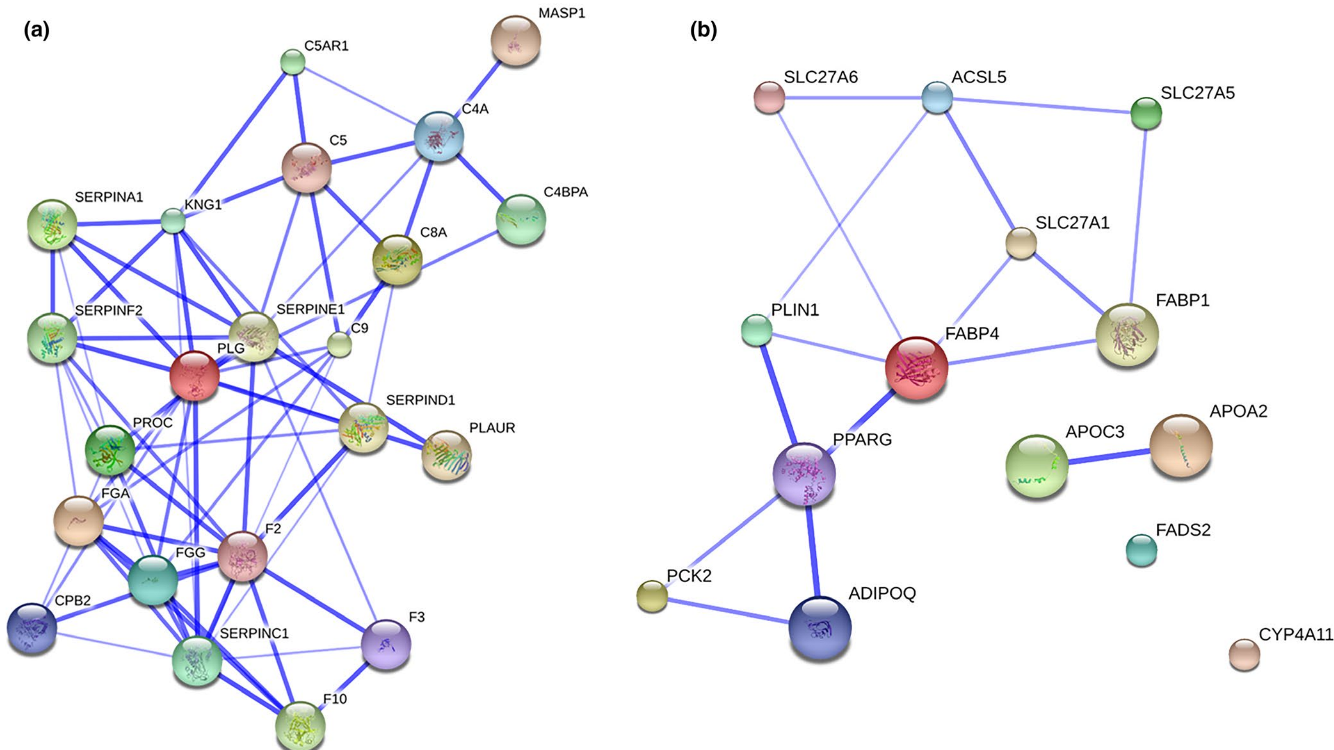

FIGURE 1 Networks of differentially expressed genes (DEGs) involved in the complement and coagulation cascades (a) and the PPAR signalling pathway (b). DEGs were categorized using KEGG databases

levels of the *PPARG* ($p < .001$), *FGG* ($p < .01$), *ADIPOQ* ($p < .05$) and *SERPINE1* ($p < .05$) genes were higher in the IMF portion than in the muscle portion (Figure 2).

4 | DISCUSSION

Castration is widely known to positively affect beef quality. To identify the genes and pathways that contribute to improved beef quality, our previous studies compared gene expression in the *longissimus* muscles and livers of Korean cattle (Baik, Nguyen, Jeong, Piao, & Kang, 2015; Baik, Vu, Piao, & Kang, 2014; Jeong et al., 2013). These studies showed that castration significantly improved beef quality and that this improvement was accompanied by correlated changes in lipid metabolic gene expression in the LT of cattle (Baik et al., 2014, 2017; Bong et al., 2012). However, the detailed mechanism of beef quality improvement after castration remains unclear because qPCR and microarray analysis have the limitation of detecting only known and selected genes. RNA-Seq is a sequence-based method that overcomes the limitations of existing technologies (Wang, Bower, et al., 2009). This study is the first attempt to apply RNA-Seq technology to the determination of the transcriptomic changes following the castration of Korean cattle bulls and the identification of the novel genes or pathways related to beef quality in LT tissues.

We obtained a total of 640 DEGs (307 up-regulated in bulls and 333 in steers; FDR < 0.05 and $|\log_2 FC| \geq 1.5$) from the LT tissues of bulls and steers. Based on the KEGG pathway analysis of DEGs, we found that the PPAR signalling pathway, a previously known pathway involved in IMF deposition and beef quality, changed following castration (Table 2). Additionally, we found that unexpected pathways, *that is* the complement and coagulation cascade pathways, were altered following castration.

The PPAR signalling pathway is composed of three different PPAR isotypes, including PPARα, PPARβ/δ and PPARγ. PPAR forms a heterodimer with retinoid X receptor, which acts as a transcription factor for genes related to energy homeostasis, cell differentiation and proliferation, and all PPARs enhance anti-inflammatory effects (Wahli & Michalik, 2012). In our study, 14 DEGs (*PPARG*, *FABP4*, *PLIN1*, *ADIPOQ*, etc.) were involved in the PPAR signalling pathway (Table 2). PPARγ is well known as an important regulator of adipocyte differentiation, lipid metabolism, glucose metabolism and inflammation. PPARγ is activated by fatty acids and controls the expression of target genes, including *leptin* and *ADIPOQ* (Ahmadian et al., 2013; Michalik et al., 2006). In agreement with the results of this study, our previous studies have shown higher expression of *PPARG* and *FABP4* in steers than in bulls (Bong et al., 2012; Park, Kang, et al., 2018). Fatty acid-binding protein 4 plays a role in fatty acid transportation, and this protein is regarded as an indicator of intramuscular adipocyte content, as it is mainly expressed in adipocytes (Jurie et al., 2007; Taniguchi et al., 2008). In IMF tissue from steers, castration increased the expression of bta-*let*-7i, which is a miRNA that up-regulates *PPARG* and *PLIN1* (Zhang et al., 2017).

TABLE 3 Comparison data of RNA sequencing and qPCR to validate DEGs involved in complement and coagulation cascades

Gene name	Gene symbol	RNA sequencing (*n* = 3)			Real-time PCR (*n* = 10)	
		FC(steer/bull)	*p*-value	FDR	FC(steer/bull)	*p*-value
Up-regulated genes in steers						
Coagulation factor III, tissue factor	*F3*	4.68	<.001	0.001	3.52	.001
Serpin family E member 1 (plasminogen activator inhibitor 1)	*SERPINE1 (PAI−1)*	11.7	<.001	<0.001	3.48	.04
Plasminogen activator, urokinase receptor	*PLAUR*	4.48	<.001	0.01	2.00	.02
Complement C4A	*C4A*	3.20	<.001	0.002	1.74	.09
Complement C5a receptor 1	*C5AR1*	4.26	.001	0.03	2.62	.002
Down-regulated genes in steers						
Fibrinogen alpha chain	*FGA*	0.01	<.001	<0.001	0.05	.15
Fibrinogen gamma chain	*FGG*	0.04	<.001	<0.001	0.13	.14
Serpin family A member 1	*SERPINA1*	0.001	<.001	<0.001	0.06	.20
Serpin family C member 1	*SERPINC1*	0.06	<.001	<0.001	0.13	.12
Mannan-binding lectin serine peptidase 1	*MASP1*	0.19	<.001	<0.001	0.21	.003

Abbreviation: FDR, False discovery rate.

Perilipin 1 regulates lipid storage as a component protein of the lipid droplet structure. In humans and pigs, PLIN1 is exclusively expressed in adipocytes and not in myofibres (Gandolfi et al., 2011; Phillips et al., 2005). Adiponectin is one of the adipokines, a group of adipocyte-derived hormones that includes leptin. Transcription factors including PPARγ, C/EBPα (*CCAAT/enhancer-binding protein alpha*) and sterol regulatory element binding protein activate the expression of adiponectin in adipocytes (Liu & Liu, 2010). Adiponectin then spreads through the bloodstream and promotes fatty acid oxidation, glucose utilization, insulin sensitivity and the anti-inflammatory response (Gnacinska, Malgorzewicz, Stojek, Lysiak-Szydlowska, & Sworczak, 2009; Liu & Liu, 2010; Yamauchi et al., 2002). Wang, Bower, et al. (2009) reported that the *longissimus* muscle of Wagyu × Hereford crossbreed cattle had a higher IMF content than did that of a Piedmontese × Hereford crossbreed, and higher expression levels of *PPARG* and *ADIPOQ* were observed in the *longissimus* muscle of the Wagyu × Hereford crossbreed than in the longissimus muscle of the Piedmontese × Hereford crossbreed at 30 months of age (Wang, Bower, et al., 2009). The aforementioned genes (*PPARG*, *FABP4*, *PLIN1* and *ADIPOQ*) play important roles in lipid metabolism, including in adipogenesis, fatty acid transportation and lipid storage, and these genes are used as proxies for IMF deposition (Albrecht et al., 2011; Jeong et al., 2013; Park, Kang, et al., 2018). Collectively, our study confirms that PPAR signalling is important for regulating bovine IMF deposition.

The complement and coagulation cascade pathways were the most enriched pathways identified in the KEGG pathway analysis of the DEGs in this study. Both the complement and coagulation pathways are essential systems involving proteolytic processes that maintain homeostasis. The complement system is composed of several complement factors that participate in both innate immunity and adaptive immunity against invaders (Markiewski, Nilsson, Ekdahl, Mollnes, & Lambris, 2007). The coagulation system consists of a series of proteolytic processes that result in fibrinogen being converted into fibrin and in fibrin then preventing bleeding by forming a blood clot with blood components (Markiewski et al., 2007).

In this study, several DEGs of the complement and coagulation cascade pathways were detected in the LT. We noted up-regulation of *SERPINE1* and *F3* gene expression following castration. Adipocytes play a role not only in lipid storage but also as an endocrine organ that secretes many signalling compounds, including adipokines (leptin, adiponectin, resistin, etc.), cytokines (interleukin-6, tumour necrosis factor α, transforming growth factor β, etc.) and several complement and coagulation factors encoded in *C3*, *C5*, *SERPINE1* (also known as PAI-1) and *F3* (Darvall, Sam, Silverman, Bradbury, & Adam, 2007; Kershaw & Flier, 2004; Schaffler & Scholmerich, 2010). When an animal is in an obese state, mature hypertrophied adipocytes suffer oxygen deficiency, called hypoxia, and these adipocytes then secrete pro-inflammatory cytokines, such as interleukin-6 and other chemokines, via the hypoxia-inducible factor (Pugia, 2015). Macrophages are then accumulated around adipocytes and stimulate the adipocytes to secrete pro-inflammatory cytokines, complement components and coagulation factors (Darvall et al., 2007; Pugia, 2015; Schaffler & Scholmerich, 2010). In this study, we also found that the mRNA levels of *C5AR1* were higher in steers than in bulls. In obese adipose tissue, oxidative stress leads to complement activation, along with increased complement receptor expression (Phieler, Garcia-Martin, Lambris, & Chavakis, 2013; Pugia, 2015). C5AR1, which is a receptor of complement C5a, was up-regulated in obese adipose tissue, and up-regulation of C5AR1 was implicated as a contributor to the accumulation of pro-inflammatory macrophages

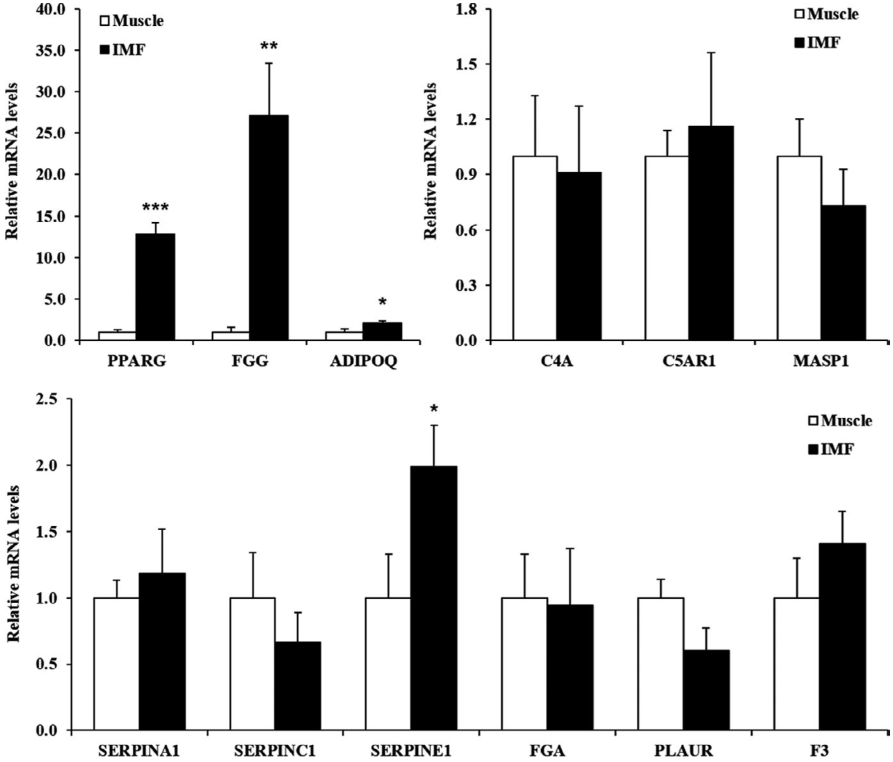

FIGURE 2 Comparison of mRNA expression levels between muscle and intramuscular fat (IMF) portions of *longissimus thoracis* tissue in Korean cattle steers (n = 10 animals/group). The mRNA expression levels were determined through qPCR, and the results were normalized to a reference gene (ribosomal protein lateral stalk subunit P0). mRNA expression levels of muscle were normalized to 1.0. Values presented are mean + *SE*. $^*p < .05$; $^{**}p < .01$; $^{***}p < .001$. *PPARG*: *peroxisome proliferator-activated receptor gamma*; *FGG*: *fibrinogen gamma chain*; *ADIPOQ*: *adiponectin, C1Q, and collagen domain containing; C4A*: *complement C4A*; *C5AR1*: *complement C5a receptor 1*; *MASP1*: *mannan-binding lectin serine peptidase 1*; *SERPINA1*: *serpin family A member 1*; *SERPINC1*: *serpin family C member 1*; *SERPINE1*: *serpin family E member 1*; *FGA*: *fibrinogen alpha chain*; *PLAUR*: *plasminogen activator, urokinase receptor*; and *F3*: *coagulation factor* III *tissue factor*

and insulin resistance (Phieler, Chung, et al., 2013). PAI-1 inhibits its plasminogen activator, which converts plasminogen to plasmin, thereby reducing fibrinolysis by plasmin (Darvall et al., 2007). PAI-1 was up-regulated in obese adipocytes and caused impaired fibrinolysis (Darvall et al., 2007; Skurk & Hauner, 2004). PAI-1 mRNA levels in human adipose tissue were increased in severe obesity (Alessi et al., 2000), and the same authors suggest that this increased PAI-1 expression could be the result of a cytokine disturbance, such as transforming growth factor-β, which accompanies insulin resistance. Our finding of increased levels of PAI-1 mRNA in steers suggests that castration may induce a cytokine disturbance in the increased adipocyte population in the LT. Tissue factor (TF; expressed from *F3* gene) plays a role in initiating the coagulation cascade by binding with coagulation factor VIIa. Up-regulation of TF mRNA levels was observed in the adipocytes of obese mice compared within those of lean mice (Samad, Pandey, & Loskutoff, 2001). Ruf and Samad (Ruf & Samad, 2015) reported that the TF signalling pathway in adipocytes contributes to the development of obesity through up-regulation of PAI-1 expression.

We observed lower *SERPINA1* mRNA levels in the LT of castrated animals than in those of non-castrated bulls. Consistent with our results, obese mice and human subjects have decreased serum levels of a1-antitrypsin (SerpinA1) (Mansuy-Aubert et al., 2013). The same authors suggested that the imbalance between *SERPINA1* and neutrophil elastase causes the development of obesity and is related to inflammation and insulin resistance. Little information is available about the expression of the SERPINA1 gene in adipose or muscle tissue. Thrombin promoted diet-induced obesity through fibrin-driven inflammation in mice (Kopec et al., 2017), and inhibiting thrombin activity alleviates the development of obesity. We observed lower transcript levels of the SERPINC1 gene (which encodes antithrombin) in the LT of castrated animals compared within that of non-castrated bulls. Thus, the decreased expression of antithrombin-encoding genes in the LT of castrated animals may be associated with the increased IMF deposition.

Collectively, our results along with those from the literature suggest that changes in the complement and coagulation cascade pathways might be associated with higher IMF deposition after castration due to obesity and subsequent inflammation in the LT. The transcriptomic changes in the LT of castrated animals may represent the alteration of processes related to the coagulation cascade through TF expression and impairment of the fibrinolytic system, as suggested in human obese study (Vilahur, Ben-Aicha, & Badimon, 2017).

To understand the effects of castration on beef quality and their associations with transcriptome expression at the conventional slaughter

ages of Korean cattle, bulls (26 months of age) and steers (32 months of age) of typical slaughter ages were used. Cattle age affected beef quality (Bartoň, Bureš, Kott, & Řehák, 2011), and it may also affect transcriptome levels. This aspect limits the interpretations of our results.

In conclusion, we examined the transcriptomic changes in bovine LT through RNA-Seq analysis following the castration of bulls. PPAR signalling and the complement and coagulation cascade pathways were most enriched in the DEGs, and the most significant DEGs were related to the complement and coagulation cascade pathways, adipocyte differentiation, and lipid metabolism. Our findings suggest a potential role of complement and coagulation factors in IMF deposition and provide new insights into the effects of castration on LT tissue and beef quality. Through application of RNA-Seq to transcriptomic analysis, our knowledge of the effects of castration on beef quality may be expanded to include the novel biological roles of previously unidentified systems, such as the complement and coagulation cascade pathways in the LT.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ANIMAL WELFARE STATEMENT

All experimental procedures were performed in accordance with the Animal Experimental Guidelines provided by the Seoul National University Institutional Animal Use and Care Committee, Republic of Korea. The experimental protocol was approved by the Seoul National University Institutional Animal Use and Care Committee (SNU-151221-4). The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

DATA AVAILABILITY STATEMENT

The data sets generated or analysed during this study are available from the corresponding author on reasonable request. The RNA-Seq data obtained from this study have been submitted to the NCBI Gene Expression Omnibus (GEO) under accession number GSE95789 (http://www.ncbi.nlm.nih.gov/geo/).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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