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Differential expression of extracellular matrix and integrin genes in the *longissimus thoracis* between bulls and steers and their association with intramuscular fat contents

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ABSTRACT

This study was performed to compare expression of genes for extracellular matrix (ECM) components, ECM degrading factors, and integrin subunits in the *longissimus thoracis* (LT) between bulls and steers. Steers had lower ($P < 0.05$) ECM component collagen type 1 α1 and collagen type 3 α1 mRNA levels than did bulls, but they had higher ($P < 0.05$) thrombospondin 1 mRNA and protein levels. Steers had higher ($P < 0.01$) matrix metalloproteinase (MMP) 9 mRNA levels than did bulls. Steers had higher ($P < 0.05$) integrin α5 mRNA levels but lower ($P < 0.05$) integrin β6 mRNA and protein levels; however, expression levels of several other integrin subunits were not different between steers and bulls. MMP9 mRNA levels were positively correlated ($P < 0.05$) with intramuscular fat content in bull group. In conclusion, these results demonstrate that castration has moderate effects on expression of ECM components, ECM degrading factors, and integrin subunit genes in the LT.

1. Introduction

Castration significantly increases the marbling score (MS) and intramuscular fat (IMF) deposition, and thus improves the quality grade (QG) of beef (Bong et al., 2012; Park et al., 2002). Our previous studies showed that activating adipogenesis and lipogenesis is important for increasing IMF deposition following castration (Baik et al., 2017; Baik, Nquyen, Jeong, Piao, & Kang, 2015; Bong et al., 2012; Jeong, Kim, Nguyen, Lee, & Baik, 2013; Jeong, Kwon, Im, Seo, & Baik, 2012).

Fibrogenesis is another mechanism regulating IMF deposition. Du et al. (2013) suggested that muscle cells, adipocytes, and fibroblasts are all derived from the same pool of mesenchymal progenitor cells, which are present in mature muscle, although they are abundant during early developmental stages. The majority of these cells undergo myogenic differentiation, but a significant proportion differentiates into common progenitor cells, committed to either adipocytes and fibroblasts or so-called fibro/adipogenic progenitor cells, which are located in the extracellular matrix (ECM) of muscle fibers (Du et al., 2013). The same authors suggested that enhancing the adipogenic differentiation of these progenitor cells increases IMF, whereas fibrogenic differentiation stimulates the synthesis of ECM components or connective tissue in muscle. Japanese Wagyu muscle has higher IMF and collagen contents compared with those of Angus muscle, and this is accompanied by increased adipogenesis and fibrogenesis in Wagyu muscle compared with Angus muscle (Duarte et al., 2013). Castration may affect the regulation of fibrogenic gene expression. However, little is known about the molecular changes associated with fibrogenesis in the *longissimus dorsi* (LM) following castration of bulls.

Marbling adipocytes and connective tissue, such as collagen and fibronectin, are present in the ECM of the LM (Duarte et al., 2013). The amount of IMF deposition and the collagen contents are higher in Wagyu muscle than in Angus muscle (Duarte et al., 2013). Thrombospondin 1 (THBS1) is a matricellular ECM protein that interacts with other ECM proteins, such as collagens (Mumby, Raugi, & Bornstein, 1984). Thrombospondin 1 is also an adipokine that is highly expressed in obese human subjects (Varma et al., 2008). No study has investigated THBS1 during adipogenesis of cattle. Changes in the expression of genes for ECM components may affect the degree of marbling following castration.

Matrix metalloproteinase (MMP) family members are essential

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enzymes to degrade the ECM, and they are considered to play important roles in adipogenesis and fibrogenesis in rodent studies (Bouloumié, Sengenès, Portolan, Galitzky, & Lafontan, 2001; Chavey et al., 2003; Sternlicht & Werb, 2001). Among MMP family members, MMP9 is implicated as an important regulator of fat deposition (Bouloumié et al., 2001; Bourlier et al., 2005). Matrix metalloproteinase 13 degrades collagen (López-Rivera et al., 2005). In their review, Christensen and Purslow (2016) suggested important roles for MMP in the regulation of myogenesis, fibrogenesis, and adipogenesis, thereby possibly affecting meat quality in domestic animals. Matrix metalloproteinases are downregulated by tissue inhibitor of metalloproteinase (TIMP) family members (Balcerzak, Querengesser, Dixon, & Baracos, 2001; Parsons, Watson, Brown, Collins, & Steele, 1997), and TIMP is implicated in adipose tissue remodeling (Fenech, Gavrilovic, & Turner, 2015). Matrix metalloproteinases and TIMP may have functions in regulating IMF deposition and fibrogenesis in cattle.

The integrin family of adhesion receptors is comprised of cell surface proteins that mediate adhesion to the ECM and cell-cell interactions (Hynes, 2002). Integrins are heterodimeric transmembrane proteins comprised of α and β subunits (Legate, Wickström, & Fässler, 2009). Extracellular matrix components, including fibronectin and THBS1, exert their biological effects through specific cell surface receptors, such as integrins (Dean, Birkenmeier, Rosen, & Weintraub, 1991; Lawler, 2000). One study reported that integrins regulate the proliferation and differentiation of human adipose-derived stem cells (Morandi et al., 2016). Integrin signaling through ECM components may help control adipogenesis and fibrogenesis in the LM of cattle.

However, limited information is available on the role of ECM-associated factors in IMF deposition in the LM. Furthermore, little is known about the molecular changes associated with fibrogenesis, ECM components, ECM degrading factors (MMP and TIMP), and integrins in the LM following castration of bulls. We hypothesized that in addition to adipogenesis, castration affects gene expression of ECM components and its associated genes, contributing to IMF deposition, MS, and QG of beef. This study was performed to understand the transcriptional changes in fibrogenic factor, ECM component, ECM degrading factor, and integrin receptor genes in the LM following castration of bulls. The relationships between gene expression levels and IMF contents were also investigated.

2. Materials and methods

All experimental procedures involving animals were approved by the Chonnam National University Institutional Animal Use and Care Committee (CNUIAUCC: CNU IACUC-YB-2010-9), Republic of Korea. The experiments were conducted in accordance with the Animal Experimental Guidelines of CNUIAUCC.

2.1. Animals and tissue samples

In this study, we used *longissimus thoracis* (LT) samples to determine the expression levels of genes in all tissues in 10 bulls and 10 Korean cattle steers from a previous study (Bong et al., 2012). The feeding method was described previously, and the carcass characteristics have been reported by our laboratory (Bong et al., 2012). Of the 20 steers, we used the 10 with the highest marbling scores (6–9) on a scale of 1–9, with 1 being the lowest and 9 the highest marbling score, to examine differential gene expression by marbling score. We used the same animals as Jeong et al. (2013) except two steers because of a lack of tissue samples. The mean marbling score (1.1 vs. 7.2 in bulls and steers) and QG were significantly higher ($P < 0.001$) in steers compared to bulls. The mean slaughter age and mean carcass weight of bulls and steers were 20 and 28 months, and 347 kg and 414 kg, respectively.

Intramuscular fat content was measured following the procedure of Folch, Lees, and Sloane Stanley (1957). Briefly, LT tissues were ground to a fine powder and homogenized in a 2:1 chloroform-methanol mixture (vol/vol). The fat-containing solvents were evaporated, and the fat content was measured. Nine steer samples were analyzed for IMF contents due to a lack of tissue sample of one steer. Steer LT had 4.0-fold greater ($P < 0.001$) IMF content (16.6 ± 1.83%) compared with bull LT (4.2 ± 0.53%).

We separated the muscle and IMF portions from the LM of the steers to evaluate the effect of IMF content on gene expression in the LT, as described previously (Bong et al., 2012).

2.2. RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from tissues using TRIzol Reagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's instructions. RNA concentration was measured using a NanoPhotometer (Implen GmbH, Schatzbogen, München, Germany). The integrity of total RNA was initially verified through ethidium bromide staining of the 28S and 18S agarose gel electrophoresis bands. RNA integrity was also verified using a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA): an RNA integrity number (RIN) ≥ 8.0 was considered acceptable. RNA was stored at −70 °C until analysis.

Total RNA was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA), according to the manufacturer's instructions. Reverse transcription was conducted in a 10-μL total reaction volume that contained 2 μg RNA template, 2 μL of 5 × iScript Reaction Mix, 0.5 μL of iScript reverse transcriptase, and 2.5 μL of nuclease-free water. The thermal parameters were: 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min.

The qPCR was performed as reported previously (Bong et al., 2012; Jeong et al., 2013) using QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, Valencia, CA, USA). We followed the “Minimum Information for Publication of Quantitative Real-Time PCR Experiments” (MIQE) guidelines for qPCR as much as possible (Bustin et al., 2009). All qPCR analyses were conducted in a 25-μL total reaction volume that contained 20 ng cDNA, 12.5 μL SYBR Green RT-PCR Master Mix, and 1.25 μL of 10 μM primers. The thermal cycling parameters were: 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. Primer information is presented in Supplementary Table 1. We used two different exons for forward and reverse primers to prevent amplification of the DNA template. We indicated the melting temperatures (Tm) of all primers to Supplementary Table 1. The Tms of all of the primers were 57.0–61.5 °C. Thus, an annealing temperature of 55 °C was used for amplification of all of the genes, resulting in a single major peak in all cases. We tested primer amplification efficiency for all genes according to Bustin et al., 2009. Briefly, cDNA was serially diluted, qPCR was performed as described above, and a calibration curve was generated as follows: the logarithm of the cDNA template concentration was plotted on the *x*-axis and the quantitation cycle (Cq) was plotted on the *y*-axis. PCR efficiency was determined from the slope of the linear portion of the log calibration curve; PCR efficiency = $10^{-1/\text{slope}} - 1$. Primer efficiency for all genes was 90–110%, which is acceptable for qPCR (Supplementary Table 1). We used a similar primer efficiency test method in a previous study (Ahn et al., 2014). The ΔΔCT method was used to determine the relative fold change in gene expression (Livak & Schmittgen, 2001). In this study, we evaluated whether β-actin, ribosomal protein lateral stalk subunit P0 (RPLP0), and 18s RNA gene are suitable reference genes. β-actin expression was generally uniform in the LT between bulls and steers, whereas RPLP0 expression was uniform in both the muscle and IMF portions of the LT. Therefore, β-actin was used as the reference gene in the LT, and RPLP0 was used in comparisons of the IMF and muscle. We used β-actin as a reference gene in the LT in two previous studies of bulls and steers (Bong et al., 2012; Jeong et al., 2013). Ribosomal protein lateral stalk subunit P0 is reportedly one of the most stable reference genes in bovine adipose tissue, muscle, and mammary glands (Bonnet, Bernard,

Bes, & Lerous, 2013).

2.3. Western blot analysis

Of the 10 bulls and 10 steers, 4 bulls and 4 steers with slaughter ages, marbling scores, and carcass weights similar to the mean values were used for Western blot analysis. Samples were homogenized in a Polytron homogenizer for 30 s in cold Pro-PREP protein extraction solution (Intron Biotechnology, Seoul, Korea), and the homogenized samples were incubated at 4 °C for 30 min. Samples were centrifuged (13,000 rpm for 30 min at 4 °C), and the protein content of each supernatant was determined using the bicinchoninic acid assay (BCA) protein assay kit (Pierce, Rockford, IL, USA). Total proteins were prepared for Western-blot analysis by boiling in 5 × sample buffer (50 mM Tris, 2% sodium dodecyl sulfate, 5% glycerol, and 10% 2-mercaproethanol; pH 6.8). The homogenized proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 6% polyacrylamide separating gel and a 5% stacking gel for integrin β6 (ITGB6) (97 kDa) and THBS1 (170 kDa). The proteins were transferred to wet polyvinylidene fluoride membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA) using a Mini Trans-Blot Cell (Bio-Rad Laboratories Inc., Hercules, CA, USA), blocked with 1 × TBS/0.1% Tween 20 and 5% nonfat dried milk for 1 h at room temperature, and incubated with the appropriate antibody overnight at 4 °C (ITGB6: goat polyclonal anti-ITGB6, 1:100 dilution; Santa Cruz Biotechnology, Dallas, TX, USA; THBS1: mouse monoclonal anti-THBS1, 1:200 dilution; Thermo Fisher Scientific, Waltham, MA, USA). The blots were treated with secondary horseradish peroxidase-conjugated anti-goat or anti-mouse antibodies for 1 h at room temperature (1:5000 dilution; Santa Cruz Biotechnology) and developed using an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK). The blots were quantified by image processing scan analysis (Gel Doc XR; Bio-Rad Laboratories Inc., Hercules, CA, USA). Band intensities were analyzed using ImageJ software (NIH, Bethesda, MD, USA). Band densities were normalized by actin content.

2.4. Statistical analyses

All data are expressed as means + standard error. Statistical differences between bulls and steers were determined using the general linear model procedure in SAS 9.4 software (SAS Institute, Cary, NC, USA). The IMF contents were not normally distributed due to marked differences between the bull and steer groups. Thus, we performed a linear regression analysis to examine the relationship between gene expression and IMF% within bull and steer group using a SAS 9.4 software without analyzing pooled data of bulls and steers. This resulted in the following equation:

$$IMF\%_i = \beta_0 + \beta_1 Expression_i + \varepsilon_i,$$

where $IMF\%_i$ is the variable of IMF%, $Expression_i$ is the variable of gene expression level, β_0 is the intercept, β_1 is the coefficient of gene expression level, and ε_i is random error.

3. Results and discussion

3.1. Comparison of expression levels of adipogenic and fibrogenic regulator genes in the LT between bulls and steers

Castration of bulls improves the MS and beef QG of Korean cattle (Bong et al., 2012). Adipogenesis is important in IMF deposition and marbling in the LM of beef cattle (Du et al., 2013), and peroxisome proliferator-activated receptor γ (PPARG) is a central regulator of adipogenesis (Moisá et al., 2014; Rosen & MacDougald, 2006). Fatty acid binding protein 4 is upregulated by PPARG, and it plays a key role in increasing IMF deposition by transporting fatty acids (Taniguchi et al., 2008). In this study, we determined the expression levels of adipogenic

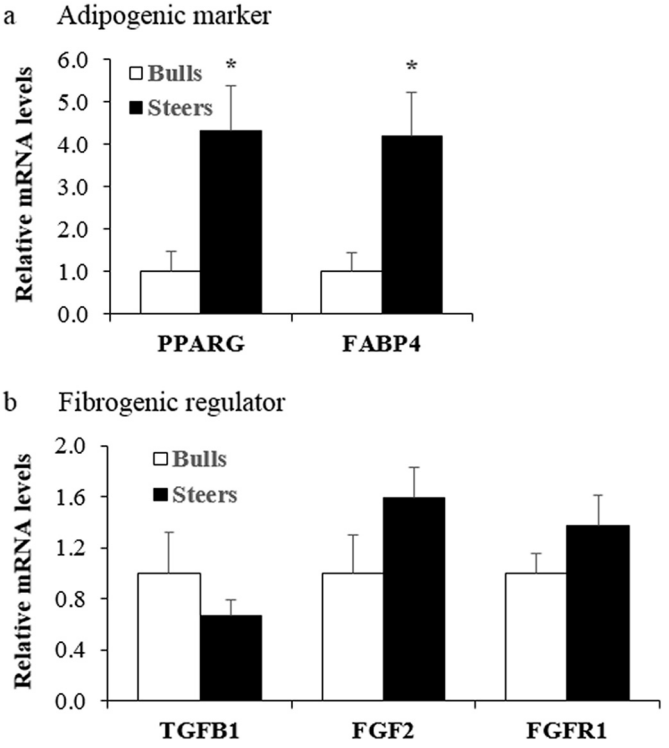

Fig. 1. Expression levels of adipogenic and fibrogenic genes in the *longissimus thoracis* (LT) between bulls and steers. mRNA levels were determined by qPCR, and the results were normalized to the β-actin as a reference gene (n = 10/group). mRNA levels of bulls were normalized to 1.0. Values are means + standard error. *$P < 0.05$. PAPRG: peroxisome proliferator-activated receptor γ; FABP4: fatty acid binding protein 4; TGFB1: transforming growth factor β1; FGF2: fibroblast growth factor 2; FGFR1: fibroblast growth factor receptor 1.

PPARG and fatty acid binding protein 4 (FABP4) genes in the LT between bulls and steers (Fig. 1a). Steers had higher ($P < 0.05$) PPARG and FABP4 mRNA levels than did bulls. The FABP4 mRNA level was markedly (57-fold) higher ($P < 0.001$) in the IMF portion than in the muscle portion (Fig. 3). We previously reported higher PPARG and FABP4 mRNA levels in steers compared to bulls (Jeong et al., 2012, 2013). The fold changes in the PPARG expression levels of bulls and steers in this study differed from those reported by Jeong et al. (2013), probably because some animals were different from those used in the previous study. However, expression pattern (higher in steers compared to bulls) was similar in both studies. Peroxisome proliferator-activated receptor γ and FABP4 expression levels were also higher in high- versus low-marbled beef (Graugnard et al., 2009). Increased cell number (hyperplasia) and cell size (hypertrophy) are involved in IMF deposition (Baik et al., 2017; Hausman et al., 2009). Albrecht et al. (2011) reported that the IMF in the LM showed both hypertrophy and hyperplasia during fattening of Japanese Black cattle, and that this was accompanied by up-regulated expression of the adipogenic PPARG and lipogenic FABP4 genes until 22 months of age. Thus, the higher PPARG and FABP4 mRNA levels in this study may be due to a greater number of adipocytes as well as larger adipocyte cell size in muscle samples with more IMF following castration. Animal age may influence IMF deposition and marbling score. The IMF contents increased as animals aged in several cattle breeds including German Angus, Galloway, Holstein-Friesian, and Belgian Blue (Albrecht, Teuscher, Ender, & Wegner, 2006). This age-dependent increase in IMF content may affect adipogenic gene expression. Japanese Black cattle and Holstein showed age-dependent adipogenic gene expression in the LM (Albrecht et al., 2011): both PPARG and FABP4 mRNA levels showed a peak expression at 22 months of age among several biopsy time points (10, 14, 18, 22, 26 months of age), and expression levels of both genes were lower at

Table 1
Regression analysis between gene expression level in the *longissimus thoracis* and intramuscular fat content (%) in Korean cattle.

Gene name (Symbol)	Bulls (n = 10)			Steers (n = 9)[a]		
	Coefficient	*P*-value	R^2	Coefficient	*P*-value	R^2
Peroxisome proliferator-activated receptor γ (PPARG)	− 0.228	0.228	0.199	− 0.087	0.747	0.023
Fatty acid binding protein 4 (FABP4)	0.214	0.335	0.133	0.209	0.423	0.132
Transforming growth factor β1 (TGFB1)	− 0.068	0.840	0.006	1.408	0.505	0.093
Fibroblast growth factor 2 (FGF2)	− 0.371	0.252	0.182	− 0.331	0.726	0.027
Fibroblast growth factor receptor 1 (FGFR1)	− 1.313	0.008	0.660	− 0.895	0.372	0.162
Collagen type 1 α1 (COL1A1)	− 1.064	0.151	0.271	− 4.736	0.200	0.303
Collagen type 3 α1 (COL3A1)	− 0.287	0.123	0.306	− 4.850	0.096	0.455
Lysyl oxidase (LOX)	0.097	0.742	0.017	1.926	0.004	0.832
Thrombospondin 1 (THBS1)	0.101	0.880	0.003	0.046	0.969	0.000
Syndecan 4 (SDC4)	− 0.058	0.956	0.000	0.043	0.988	0.000
Matrix metallopeptidase 9 (MMP9)	1.764	0.047	0.409	2.397	0.064	0.408
Matrix metallopeptidase 13 (MMP13)	− 1.080	0.278	0.145	1.161	0.319	0.141
Tissue inhibitor of metallopeptidase 1 (TIMP1)	− 0.933	0.082	0.370	− 1.241	0.098	0.452
Tissue inhibitor of metallopeptidase 3 (TIMP3)	− 0.709	0.060	0.417	− 0.482	0.159	0.354
Integrin α1 (ITGA1)	− 0.240	0.566	0.043	− 0.961	0.500	0.067
Integrin α3 (ITGA3)	0.064	0.867	0.004	− 4.327	0.097	0.454
Integrin α4 (ITGA4)	− 0.299	0.461	0.070	− 1.678	0.490	0.071
Integrin α5 (ITGA5)	0.001	0.999	0.000	− 1.484	0.319	0.141
Integrin α11 (ITGA11)	− 0.723	0.515	0.089	− 0.918	0.800	0.025
Integrin αV (ITGAV)	− 0.024	0.978	0.000	1.117	0.645	0.032
Integrin β1 (ITGB1)	0.187	0.877	0.003	2.371	0.569	0.048
Integrin β6 (ITGB6)	2.669	0.211	0.188	− 10.521	0.528	0.059

[a] Nine steers were subjected to regression analysis because the IMF content of one steer was not determined due to a lack of tissue samples.

26 months compared with 22 months. The same authors suggest that the higher transcriptional activity of adipogenic genes may be due to a higher number of differentiating cells during growth and that the already stored IMF contents may not be directly related to gene expression levels. In the current study, regression analysis indicated no significant relationship of PPARG and FABP4 mRNA levels with IMF% in steer group (Table 1). This may be due to old slaughter age (28 months) of steers, in which time low number of differentiating cells is present in the LT. Another possibility for no relationship between PPARG mRNA levels with IMF% is that we used 9 steers with narrow range of MS (6–9) in this study. In previous study with 41 steer group (MS range 2–9; Jeong et al., 2012), we observed significant correlation ($r = 0.40$, $P < 0.01$) of PPARG mRNA levels with IMF% (Jeong et al., 2013).

Du et al. (2013) suggested that muscle cells, adipocytes, and fibroblasts are derived from the same pool of mesenchymal progenitor cells, and that mesenchymal progenitor cells are present in mature muscle, although they are abundant during early developmental stages. The majority of these cells undergo myogenic differentiation, but a significant proportion differentiate into common progenitor cells committed to both adipocytes and fibroblasts, which are located in the ECM of muscle fibers (Du et al., 2013). Du et al. (2013) suggested that enhancing adipogenic differentiation of these progenitor cells increases the IMF level, whereas fibrogenic differentiation stimulates the synthesis of connective tissue in muscle. Wagyu cattle are genetically predisposed to produce extremely high marbling (Gotoh, Takahashi, Nishimura, Kuchida, & Mannen, 2014). Duarte et al. (2013) reported a higher IMF content in the sternomandibularis muscle of Wagyu compared to Angus cattle. Moreover, the expression of adipogenic and fibrogenic genes was higher in Wagyu than in Angus cattle, revealing enhanced adipogenesis and fibrogenesis in the muscle of the former. To evaluate the involvement of fibrogenesis in increased MS following castration, we determined the expression levels of the genes encoding fibrogenic regulator transforming growth factor β1 (TGFB1), fibroblast growth factor 2 (FGF2), and fibroblast growth factor receptor 1 (FGFR1) in the LT between bulls and steers (Fig. 1b). Transforming growth factor β1, FGF2, and FGFR1 mRNA levels were not different between bulls and steers. Transforming growth factor β1 is a key regulator of fibrogenesis (Liu & Gaston Pravia, 2010; Miao et al., 2015). Among the three TGFB isoforms, TGFB1, which is abundantly expressed in fibroblasts, is most closely associated with fibrogenesis (Ghosh et al., 2005). In addition to TGFB signaling, the FGF family of proteins are also important in fibrogenic regulation. Among them, FGF2 is closely related to fibrogenesis (Kashiwakura & Takahashi, 2005). All of these fibrogenic regulators are involved in fibrogenic regulation during embryonic and fetal stages (Du et al., 2013). Interestingly, regression analysis revealed negative association ($P < 0.01$) of FGFR1 mRNA levels with IMF% in bull group, but not in steer group (Table 1). These results raise the possibility that FGFR1 may have a function in adipogenesis and fibrogensis in bull group.

Overall, these results confirm that castration was accompanied by increased adipogenic gene expression. However, castration did not affect the expression of fibrogenic regulator genes in the LT, such as TGFB1, FGF2, and FGFR1. Therefore, castration may induce adipogenesis without affecting fibrogenesis. Our results suggest that castration does not directly regulate the expression of these fibrogenic genes in the adult stage of Korean cattle, and that fibrogenic regulators are not significant factors in IMF deposition following castration at slaughter age. Our results are not consistent with a previous report of higher expression of adipogenic and fibrogenic genes in high-marbled Wagyu than in Angus cattle (Duarte et al., 2013). Taken together, a non-genetic factor (castration model in this study) and a genetic factor (breed type: e.g., Wagyu vs. Angus cattle) may have differentially affected adipogenesis and fibrogenesis in the LM. Further study is warranted to identify the role of adipogenesis and fibrogenesis in regulating marbling and ECM remodeling by both non-genetic and genetic factors.

3.2. Comparison of ECM component gene expression levels in the LT between bulls and steers

Intramuscular fat deposition occurs between muscle fiber bundles where ECM components are located. A study of Japanese Black cattle reported that the IMF deposition inside perimysium desorganizes the intramuscular connective structure (Nishimura, 2010). Collagens are major components of the ECM in muscle, and collage types 1 and 3 are abundant in muscle connective tissue (Lepetit, 2008). To determine the effect of castration on the gene expression of genes encoding ECM components, we evaluated expression levels of the collagen types 1 and 3 genes in the LT between bulls and steers. Steers showed lower mRNA

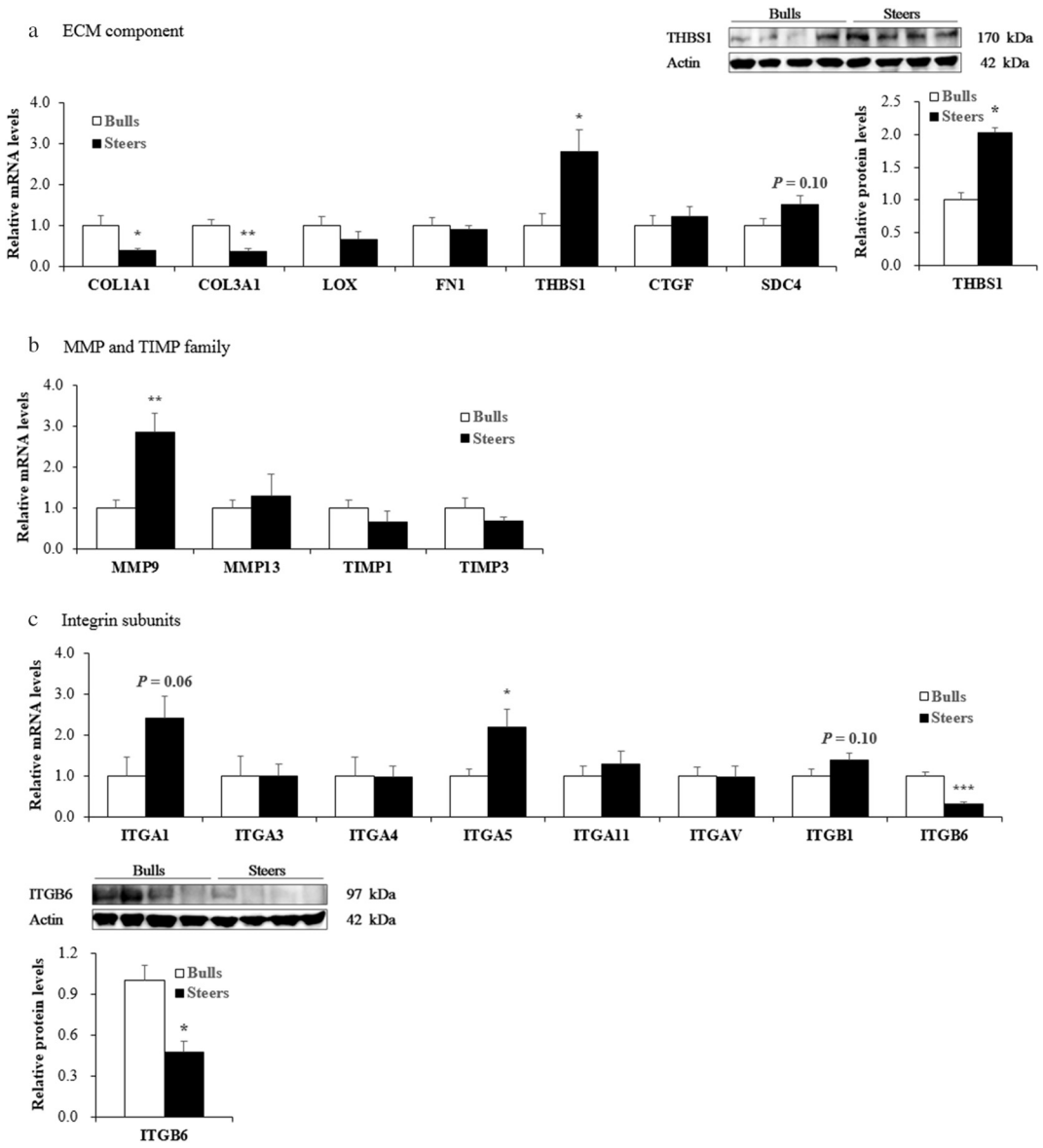

Fig. 2. Expression levels of genes associated with extracellular matrix components in the *longissimus thoracis* between bulls and steers. mRNA levels were determined by qPCR, and the results were normalized to the β-actin as a reference gene (n = 10/group). Protein levels were determined by Western blot analysis and normalized to actin levels (n = 4/group). Uncropped Western blots were provided in Supplementary Fig. 1. mRNA and protein levels in bulls were normalized to 1.0. Values are means + standard error. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. COL1A1: collagen type 1 α1; COL3A1: collagen type 3 α1; LOX: lysyl oxidase; FN1: fibronectin 1; THBS1: thrombospondin 1; CTGF: connective tissue growth factor; SDC4: syndecan 4; MMP9: matrix metallopeptidase 9; MMP13: matrix metallopeptidase 13; TIMP1: tissue inhibitor of metallopeptidase 1; TIMP3: tissue inhibitor of metallopeptidase 3; ITGA1: integrin α1; ITGA3: integrin α3; ITGA4: integrin α4; ITGA5: integrin α5; ITGA11: integrin α11; ITGAV: integrin αV; ITGB1: integrin β1; ITGB6: integrin β6.

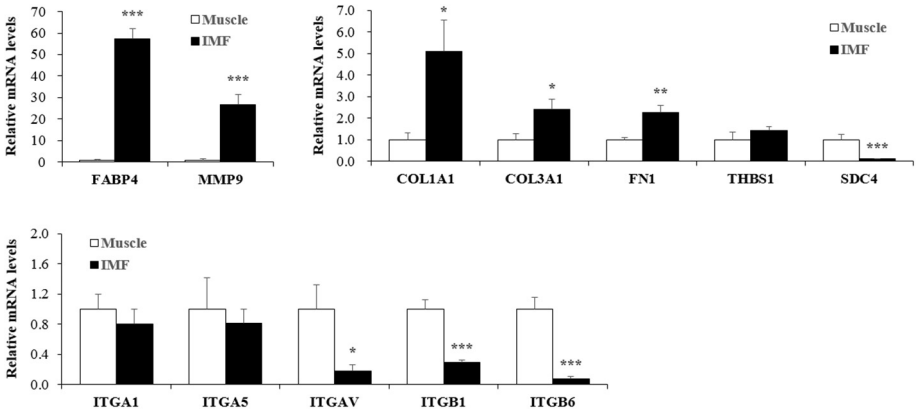

Fig. 3. Comparison of gene expression levels between muscle and the intramuscular fat portion of the *longissimus thoracis* in Korean cattle steers. The mRNA levels were determined by qPCR, and the results were normalized to the large ribosomal protein P0 as a reference gene. n = 10 animals/group for all genes, except integrin β6 (n = 10 for muscle, and n = 3 for IMF), because ITGB6 mRNA was not detected in the IMF portions of 7 animals. mRNA levels of muscle were normalized to 1.0. Values are means + standard error. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. FABP4: fatty acid binding protein 4; MMP9: matrix metallopeptidase 9; COL1A1: collagen type 1 α1; COL3A1: collagen type 3 α1; FN1: fibronectin 1; THBS1: thrombospondin 1; SDC4: syndecan 4; ITGA1: integrin α1; ITGA5: integrin α5; ITGAV: integrin αV; ITGB1: integrin β1; ITGB6: integrin β6.

levels than bulls for collagen type 1 α1 (COL1A1; $P < 0.05$) and collagen type 3 α1 (COL3A1; $P < 0.01$) genes (Fig. 2a). In one study, castrated Qinchuan cattle had lower expression levels of both COL1A1 and COL3A1 compared to those in non-castrated cattle (Zhang et al., 2011), consistent with our results. In a human study, injecting testosterone increased skeletal muscle COL3A1 gene expression (Chen et al., 2011). In our previous study, circulating testosterone levels decreased after castration of Korean cattle bulls (Bong et al., 2012). Therefore, decreased testosterone levels following castration may be a reason for the lower COL1A1 and COL3A1 mRNA transcription in steers compared to bulls. In our study, the expression levels of collagen genes were higher ($P < 0.05$) in the IMF portion than those in the muscle portion of the LT (Fig. 3), and IMF contents increased significantly following castration. Therefore, castration caused a significant decrease in collagen gene expression, despite the findings that steer LT contained higher IMF levels and that the expression levels of these genes were higher in the IMF portion than the muscle portion. Animal age may affect ECM formation. The total collagen content the m. gluteus medius increased with steer age (Girard, Aalhus, Basarab, Larsen, & Bruce, 2011). Age-dependent variation of ECM-related gene expression in the LM has been reported (Wang et al., 2009). In Wagyu × Hereford heifers, COL1A1 mRNA levels peaked both at 7 and 25 months among several biopsied sampling times (3, 7, 12, 20, 25 months of age) and at slaughter (30 months), and no difference in COL1A1 mRNA levels was observed between 20 and 30 months of age. In this study, we used bull LT of 20 months and steer LT of 28 months of age, respectively. Thus, age difference seems not to be a major factor for lower expression of COL1A1 gene in steers compared with bulls in this study. In Wagyu x Hereford heifers, COL3A1 mRNA levels were lower at 30 months compared with 20 and 25 months (Wang et al., 2009). Thus, animal age in this study may be an another factor contributing to lower expression of COL3A1 gene in steers compared with bulls, although breed and gender (male and female) differences are needed to be considered in our study and the study of Wang et al. (2009).

The collagen molecule is composed of three α-chain polypeptides (Lepetit, 2008; McCormick, 1999). Collagen cross-linking, which involves twisting of the α-chains, is catalyzed mainly by lysyl oxidase (Bornstein, Kang, & Piez, 1966; Siegel & Fu, 1976). Decreased collagen cross-linking is associated with improved meat tenderness, and collagen cross-linking is implicated in marbling development (Miao et al., 2015). To assess the effect of castration on the expression of genes associated with collagen cross-linking, we evaluated the mRNA levels of the gene encoding for lysyl oxidase in the LT between bulls and steers (Fig. 2a). Lysyl oxidase mRNA levels were not different ($P > 0.05$) between bulls and steers. Interestingly, regression analysis revealed strong positive association ($R^2 = 0.83$; $P < 0.01$) of LOX mRNA levels with IMF % in steer group, but not in bull group (Table 1). Our study indicates that castration may not significantly affect cross-linking of collagen in the LT of Korean cattle. However, collagen-cross-linking may have a role in regulating IMF deposition in steer group. Several studies have reported that collagen cross-linking is more important than the collagen content itself for determining beef tenderness (Du et al., 2013; McCormick, 1999; Miao et al., 2015).

Fibronectin is a major component of the ECM (Frantz, Stewart, & Weaver, 2010), and Duarte et al. (2013) reported that expression of the fibronectin gene was higher in the LM of highly marbled Wagyu than that of Angus cattle. We thus examined the expression levels of the fibronectin 1 gene in the LT between bulls and steers. Fibronectin 1 mRNA levels were not different between bulls and steers (Fig. 2a). Expression levels of the fibronectin 1 gene were higher ($P < 0.01$) in the IMF portion than in the muscle portion of the LT (Fig. 3). Our results indicate that castration does not significantly affect the expression of the fibronectin 1 gene, although the fibronectin 1 gene was abundantly expressed in the IMF portion.

Thrombospondin 1 is an adhesive ECM glycoprotein that is expressed abundantly in visceral adipose tissue, and its expression is elevated in obese humans (Matsuo et al., 2015; Varma et al., 2008). Inoue et al. (2013) suggested that a high-fat diet induces visceral adiposity, expansion of adipose ECM, and increased THBS1 release. This suggests that THBS1 may be involved in increased IMF deposition following castration. We thus determined the effect of castration on expression of the THBS1 gene in Korean cattle. Steers had higher ($P < 0.05$) THBS1 mRNA levels in the LT than did bulls (Fig. 2a). We also determined THBS1 protein levels by Western blotting. Steers had higher ($P < 0.05$) THBS1 protein levels than bulls in the LT, reflecting the same trend as seen for the mRNA expression levels. Thrombospondin 1 mRNA levels were not different ($P > 0.05$) in the IMF portions compared to the muscle portions of the LT (Fig. 3). Collectively, our results indicate that castration induced THBS1 expression in the LT. Further study is warranted to understand the role of THBS1 in adipogenesis and fibrogenesis of the LT in beef cattle.

Syndecan 4 is a member of the syndecan family of transmembrane-type heparan sulfate proteoglycans, and it binds to ECM proteins via their extracellular heparan sulfate side chains (Samarel, 2013). Syndecan 4 is implicated in skeletal muscle development (Cornelison et al., 2004), as well as adipocyte proliferation (Landry, Rioux, & Bensadoun, 2001). Connective tissue growth factor regulates fibrosis by binding to various growth factors, ECM proteins, and cell-surface receptors (Daniels, van Bilsen, Goldschmeding, van der Vusse, & van Nieuwenhoven, 2009). We examined the effect of castration on the expression of these ECM-associated genes. The expression levels of syndecan 4 and connective tissue growth factor genes in the LT were not different between bulls and steers (Fig. 2a). Our results show that expression levels of syndecan 4 were higher ($P < 0.01$) in muscle than those in IMF portions of the LT (Fig. 3).

Overall, we found variations in the gene expression levels of ECM components following castration: expression levels of collagen genes were lower, but THBS1 levels were higher in steers. However, expression levels of other ECM components, including lysyl oxidase, fibronectin 1, connective tissue growth factor, and syndecan 4 were not different between bulls and steers. These results indicate that castration moderately affects the expression of ECM genes.

3.3. Comparison of MMP and TIMP expression levels in the LT between bulls and steers

Members of the MMP family, which are essential enzymes for ECM degradation, play important roles in adipogenesis and fibrogenesis (Bouloumié et al., 2001; Chavey et al., 2003; Sternlicht & Werb, 2001). Among the members of the MMP family, MMP9 (also known as gelatinase B; Lijnen et al., 2001) is an important regulator of fat deposition in human adipocytes and murine cell lines (Bouloumié et al., 2001; Bourlier et al., 2005). Matrix metalloproteinase 9 is produced by mature human adipocytes and preadipocytes, and its expression is dependent on adipocyte differentiation (Bouloumié et al., 2001). Plasma MMP9 concentrations are higher in obese humans compared to normal control subjects (Derosa et al., 2008). Matrix metalloproteinase 13 (also known as collagenase 3) degrades collagen (López-Rivera et al., 2005). Matrix metalloproteinase 9 and MMP13 may be involved in adipogenesis and fibrogenesis in the LT of beef cattle. We compared the expression levels of MMP9 and MMP13 in the LT between bulls and steers (Fig. 2b). Steers showed higher ($P < 0.01$) MMP9 mRNA levels than did bulls. Regression analysis showed positive association ($P < 0.05$) of MMP9 mRNA levels with IMF% in bull group, and MMP9 mRNA levels also tended ($P = 0.064$) to correlate with IMF% in steer group (Table 1). The MMP9 mRNA level was 26.5-fold higher in the IMF portion than in the muscle portion (Fig. 3). Collectively, our data imply that MMP9 may be important for regulation of bovine IMF deposition. Hypertrophy and hyperplasia are important factors in fat deposition (Baik et al., 2017; Hood & Allen, 1973). In addition, ECM remodeling is required for fat deposition (Lijnen et al., 2001; Rupnick et al., 2002). The ECM may need to be degraded for fat to infiltrate in the area

between muscle fiber bundles. Thus, MMP9 may degrade ECM components, such as collagen types 1 and 3, to allow IMF to be deposited. Our previous study showed a significant decrease in circulating testosterone concentrations following castration of bulls (Bong et al., 2012). Androgen deprivation by administering flutamide (non-steroidal antiandrogen drug) increases MMP9 expression in the prostate of Wistar rats (Lateef et al., 2013). Thus, increased MMP9 expression following castration in this study may be in part due to decreased concentrations of testosterone. Increased IMF deposition levels following castration may also contribute to higher LT MMP9 mRNA levels in steers compared with bulls, as the mRNA levels in the IMF portions were markedly higher than those in the muscle portion of the LT. %. In this study, MMP13 mRNA levels was not different between bulls and steers.

Matrix metalloproteinases are downregulated by the TIMP family of proteins (Balcerzak et al., 2001; Parsons et al., 1997). These proteins may regulate IMF deposition and fibrogenesis in cattle. Tissue inhibitor of metalloproteinase 1 and TIMP3 mRNA levels in the LT were not different between bulls and steers. Overall, among the factors regulating ECM degradation, MMP9 may have a significant role in IMF deposition following castration of bulls.

3.4. Comparison of integrin subunit gene expression levels in the LT between bulls and steers

Integrins are the major receptor for cell adhesion to ECM proteins. They form transmembrane connections to the cytoskeleton and activate many intracellular signaling pathways (Hynes, 2002). Integrins are αβ heterodimers, and there are 18 α- and 8 β-subunits, which combine into at least 24 different heterodimers (Hynes, 2002). Specific combinations of integrin subunits regulate specific types of cellular signaling. As described above, THBS1 mRNA and protein levels were upregulated following castration in the present study. Thrombospondin 1 exerts biological effects through specific cell surface receptors, including integrins (Lawler, 2000). Integrin receptors for THBS1 include α5/β1, α3/β1, α4/β1, and αV/β3 integrins (Roberts, 1996). To determine whether changes in THBS1 expression following castration affect the expression of its downstream integrin receptor gene, we compared the expression levels of genes for THBS1 integrin receptors in the LT between bulls and steers. We found higher ($P < 0.05$) integrin α5 expression in steers compared with bulls (Fig. 2c). However, integrin α3, integrin α4, and integrin β1 expression levels were not different between bulls and steers. Therefore, we did not detect simultaneous changes in either the α- and β-subunit pairs for any of the THBS1 binding integrins. No link has been found between THBS1 and these integrins in adipocytes or skeletal muscle. Further study is needed to understand the functional roles of THBS1-integrin signaling in adipogenesis or fibrogenesis of the LT.

Four collagen binding integrins, namely α1/β1, α2/β1, α10/β1, and α11/β1, have been identified (Popova, Lundgren-Akerlund, Wiig, & Gullberg, 2007), and collagen types 1 and 3 bind to integrins α1/β1 and α11/β1 (Zeltz, Lu, & Gullberg, 2014). As described above, the COL1A1 and COL3A1 genes were downregulated following castration of bulls. To determine whether decreased expression of collagen genes following castration is linked to the expression of downstream integrin receptors, we evaluated the expression levels of genes encoding collagen binding integrin subunits in the LT between bulls and steers. Integrin α1 mRNA levels tended to increase in steers ($P = 0.06$) compared with bulls (Fig. 2c). However, integrin α11 mRNA levels were not different between bulls and steers. Therefore, we did not detect simultaneous changes in the α- and β-subunit pairs for any of the collagen binding integrins.

Fibronectin 1 interacts with 11 integrin heterodimers, including α5/β1 and αV/β6 (Leiss, Beckmann, Girós, Costell, & Fässler, 2008). To examine whether castration affects expression of fibronectin receptor genes, we determined the expression levels of the αV and β6 integrin subunit genes in the LT between bulls and steers. mRNA levels of integrin αV were not different between bulls and steers. However, integrin αV mRNA levels were lower ($P < 0.05$) in the IMF portion compared with the muscle portion of the LT. Steers showed lower ($P < 0.001$) ITGB6 mRNA levels than did bulls. Western blotting revealed that steers had lower ($P < 0.05$) ITGB6 protein levels in the LT than did bulls, reflecting the same trend as seen for the mRNA expression levels. In this study, ITGB6 mRNA levels were markedly lower ($P < 0.001$) in the IMF portion compared with the muscle portion of the LT (Fig. 3). A previous study reported that mouse skeletal muscle had abundant mRNA levels of ITGB6 (Ducceschi, Clifton, Stimpson, & Billin, 2014). In this study, we found unexpectedly lower ITGB6 mRNA and protein levels in steers compared with bulls. Transcript messages of this gene were very low in the IMF portion than in the muscle portion of the LT. Steers contained significantly higher IMF content in the LT than bulls. Therefore, the reason for the lower ITGB6 expression in steer LT may in part be due to the higher IMF portion compared with bulls.

Overall, we detected variations in integrin subunit gene expression between bulls and steers in the LT. We also found tissue-specific expression of integrin subunit genes in steers; i.e., lower mRNA levels of both integrin β1 and ITGB6 in the IMF portion compared with the muscle portion of the LT. The meaning of this differential expression between bulls and steers, and of tissue-specific expression patterns, remains unclear. Further study is warranted to understand the possible function of integrin subunits with respect to adipogenesis and fibrogenesis in the LT of cattle.

To assess the effects of castration on carcass traits and their associations with gene expression levels under the conventional slaughter conditions used by the Korean beef industry, we used bulls and steers of the typical age at slaughter (bulls 20 months and steers 28 months of age). Thus, bulls and steers used in this study were different in age and body weight. Both animal age (Albrecht et al., 2006; Girard et al., 2011) and body weight (Park et al., 2002) affected marbling score and also ECM formation. Both factors may also influence ECM-related gene expression. Currently, limited information is available for effects of age and body weight on ECM related gene expression in the LM of cattle. These aspects limit the interpretation of our findings.

4. Conclusion

Castration increased the IMF content, MS, and beef QG, and these changes were accompanied by upregulation of adipogenic gene expression without changing that of fibrogenic genes. Castration moderately affected the expression of genes encoding ECM components, ECM degrading factors, and integrin subunits, and the expression levels of some of these genes were significantly correlated with the IMF content. These results suggest that ECM components, ECM degrading factors, and integrins contribute to increased IMF content and MS in the LT following castration. Our findings suggest that these factors are involved in IMF deposition, in addition to well-known adipogenic regulators. The results of this study expand our understanding of the mechanisms regulating IMF deposition in the LT of beef cattle. This information may facilitate development of new methods of regulating IMF deposition in beef cattle.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meatsci.2017.10.008.

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