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Regional Differences of Proteins Expressing in Adipose Depots Isolated from Cows, Steers and Bulls as Identified by a Proteomic Approach

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ABSTRACT: Adipose tissue in the loin muscle area of beef cattle as a marbling factor is directly associated with beef quality. To elucidate whether properties of proteins involved in depot specific adipose tissue were sex-dependent, we analyzed protein expression of intramuscular adipose tissue (IMAT) and omental adipose tissue (OMAT) from Hanwoo cows, steers, and bulls of Korean native beef cattle by liquid chromatography-tandem mass spectrometry (LC-MS/MS)–based proteomic analysis, quantitative polymerase chain reaction (PCR) and western blot analysis. Two different adipose depots (i.e. intramuscular and omental) were collected from cows (n = 7), steers (n = 7), or bulls (n = 7). LC-MS/MS revealed a total of 55 and 35 proteins in IMAT and OMAT, respectively. Of the 55 proteins identified, 44, 40, and 42 proteins were confirmed to be differentially expressed in IMAT of cows, steers, and bulls, respectively. In OMAT of cows, steers, and bulls, 33, 33, and 22 were confirmed to be differentially expressed, respectively. Tropomyosin (TPM) 1, TPM 2, and TPM3 were subjected to verification by quantitative PCR and western blot analysis in IMAT and OMAT of Hanwoo cows, steers, and bulls as key factors closely associated with muscle development. Both mRNA levels and protein levels of TPM1, TPM2, and TPM3 in IMAT were lower in bulls compared to in cows or steers suggesting that they were positively correlated with marbling score and quality grade. Our results may aid the regulation of marbling development and improvement of meat quality grades in beef cattle. (**Key Words:** Adipose Depots, Hanwoo, Liquid Chromatography-tandem Mass Spectrometry, Marbling, Meat Quality)

INTRODUCTION

One important goal of farm animal industry is to produce high quality beef. Beef quality is normally defined by the compositional quality (lean to fat ratio) and the palatability factors such as visual appearance, smell, firmness, juiciness, tenderness, and flavor. Many studies have indicated that meat tenderness is not only affected by protein composition of muscle fibers, but also by handling and slaughtering conditions, genetic traits, and growth progress. In addition, there is some connection between tenderness and flavor through marbling of meat (Hughes et al., 2014).

Generally, marbling means the amount of intramuscular fat (Purslow, 2005; Nishimura, 2010), one of the main factors used to determine beef quality and grade in Korea. Marbling is a very important and valuable trait in the beef cattle industry (Lee et al., 2007). Previous studies have found a relationship between marbling score and percent intramuscular fat (Jeong et al., 2012; Walter et al., 2014). Therefore, the content and distribution of body fats are of special interest for production efficiency and meat quality in farm animal industry (Gondret et al., 2008).

Contents and deposition of intramuscular fat can be

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influenced by several factors, including sex, age, breed, genotype, nutrition, and environmental factors (Maltin et al., 2003; Hausman et al., 2006). Generally, steers have more intramuscular fat, higher marbling score (Destefanis et al., 2003; Schreurs et al., 2008), and more tender meat (Peachey et al., 2002; Purchas et al., 2002) than bulls. Fat storage in cattle muscle is correlated with intramuscular fat percentage (Guo et al., 2014) because the hormonal status of beef cattle from different sex is related to meat quality characteristics, such as tenderness, fat, and protein distribution (Fritsche and Steinhart, 1998). Particularly, castration dramatically increases intramuscular fat deposition, resulting in improved beef quality in Korean cattle (Park et al., 2002).

The effects of sex steroid hormonal status, including testosterone, androgen, and estrogen, on muscle tissue and myogenic satellite cells (MSCs) have been well studied (Inoue et al., 1994; Arnold et al., 1996; Kahlert et al., 1997; Lee, 2002; Sinha-Hikim et al., 2003; Enns et al., 2008).

MSCs are adult stem cells that activate and differentiate into myotubes. Our previous studies investigated the importance of hormonal components in MSC growth and lipid droplets accumulation (Lee et al., 2011). The effect of natural hormones in adult bovine serum (cow, steer, and bull serum) in MSC proliferation was observed. We found that MSC proliferation was the highest in media supplemented with bull serum followed by cow and steer serum. Lipid droplets accumulation was increased in myotubes when MSCs were treated with 17β-estradiol (E_2) followed by E_2+testosterone or testosterone treatment alone. This may be due to various hormonal components present in the different sera. Our data have demonstrated that sex hormones are key factors affecting the proliferation of MSCs and lipid accumulation in myotubes (Lee et al., 2011).

However, factors important for the improvement of beef quality grade with sex and hormonal differences are not clearly understood *in vivo*. Therefore, identification of differentially expressed proteins in adipose depots of different sexes might be helpful in defining the functions of intramuscular fat, therefore providing strategies to control meat lipid content independently of body fat depots. The objective of this study was to determine whether proteins involved in depot specific adipose tissue properties were sex dependent. We analyzed the proteome expression of intramuscular adipose tissue (IMAT) and omental adipose tissue (OMAT) in native Hanwoo Korean beef cattle (cows, steers, and bulls) by liquid chromatography-tandem mass spectrometry (LC-MS/MS)–based proteomic analysis, quantitative polymerase chain reaction (PCR), and western blot analysis.

MATERIALS AND METHODS

Animals and sample collection

All experimental procedures involving animals were approved by the National Institute of Animal Science Institutional Animal Use and Care Committee (NIASIAUCC) and conducted in accordance with the Animal Experimental Guidelines provided by NIASIAUCC in Republic of Korea. We used adipose tissue samples of cows (n = 7), steers (n = 7), and bulls (n = 7). Tissue samples were collected in three animals groups from two different adipose depots (*i.e.* intramuscular and omental). Slaughter age was approximately 31 months for all cattles. Carcass weight was 406.1±13.4, 452.6±12.3, and 490.9±13.6 kg for cows, steers, and bulls, respectively.

Gel electrophoresis and silver staining

Adipose tissues were collected from cows, steers, and bulls. Total protein isolation was performed using PRO-PREP protein extraction solution (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Proteins eluted were measured using Pierce BCA Protein Assay Kit (Thermo scientific, Rockford, IL, USA). Equal amounts of protein samples were precipitated with cold acetone. Protein pellets were dissolved in 1× sodium dodecyl sulphate (SDS) sample buffer and separated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, protein spots were visualized using protocols described in the PlusOne Silver staining kit (GE Healthcare Bio-Sciences, Uppsala, Sweden). Complete protocol was followed for analytical gels. For preparative gels, the protocol was modified. Glutaraldehyde was omitted from the sensitization step. Formaldehyde was omitted from the silver reaction step (Yan et al., 2000). Silver-stained gels were scanned (UMAX PowerLook 2100KL Imaging system, UMAX, Taiwan) and protein profiles were compared.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The resulting tryptic peptides were separated and analyzed using reversed-phase capillary high-performance liquid chromatography directly coupled to a Thermo LTQ Orbitrap mass spectrometer following the procedure described by Zuo et al. (2001) with slight modifications. Briefly, both a 0.075×20 mm trapping column and a 0.075× 120 mm resolving column were packed with C18AQ 218MS low formic acid C18 beads (5 μm in size, 200Å pore size; C18AQ, Michrom BioResources, Auburn, CA, USA) and placed in-line. Peptides were bound to the trapping column for 10 min with 2% (vol/vol) aqueous cetonitrile containing 0.1% (vol/vol) formic acid. The bound peptides were eluted with a gradient of 2% to 90% (vol/vol) acetonitrile containing 0.1% (vol/vol) formic acid at a flow rate of 0.2 μL/min. For tandem mass spectrometry, full mass scan range mode was set at m/z = 50 to 2,000 Da. After determining the charge states of the ion zoom scans, product ion spectra were

acquired in MS/MS mode with relative collision energy of 55%. Individual spectrum from MS/MS was processed using Protein discoverer 2.1 software (Thermo scientific, USA). The generated peak list files were used to query either the MSDB or the NCBI database using MASCOT program (http://www.matrixscience.com). We took into account modifications of methionine and cysteine, peptide mass tolerance at 2 Da, MS/MS ion mass tolerance at 0.8 Da, allowance of missed cleavage at 2, and charge states (namely, +1, +2, and +3). Only significant hits defined by MASCOT probability analysis were initially considered.

RNA extraction and real-time PCR analysis

Adipose tissues were collected from cows, steers, and bulls. Total RNA isolation was performed using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Briefly, total RNA levels were quantified at absorbance of 260 nm. RNA integrity was evaluated by 1.2% (w/v) agarose gel. Total RNA (2 μg amounts) was reverse-transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed with SYBR green Premix Ex Taq II (Takara, Dalian, China) using Applied Biosystems StepOne Plus Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA). The expression of β-actin was used as the endogenous control. Relative quantification analysis was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Wilting et al., 2010). Primers used in the study are listed in Table 1.

Statistical analysis

Data are reported as the mean±standard deviation of at least three independent experiments. Statistical significance was evaluated using Student's t-test. Compared to the vehicle control, $p<0.05$ were considered significant.

Table 1. Primer sequences used to generate templates for RT-PCR and real-time PCR

Gene name	Symbol	GenBank ID	Primer sequence (5'→3')	Product size (bp)
Tropomyosin 2	TPM2	NM_001010995	F: CAT TCT GCT CCG GAT ATG GT	211
			R: GCC GAG CTA CTT CAT TCT GG	
Actin, alpha 1, skeletal muscle	ACTA1	NM_174225	F: GAGCGTGGCTACTCCTTCGT	105
			R: GGTGGCCATTTCGTTCTCAA	
Aldolase A, fructose-bisphosphate	ALDOA	NM_001101915	F: CCACGCCTGTACCCAGAAAT	110
			R: CTCCGGACAGGAAGGTGATC	
Tropomyosin 1	TPM1	NM_001013590	F: GGATGCCGACCGCAAGTAT	105
			R: GCACATTTGCCTTCTGAAAGC	
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_001034034	F: CATCTCCGCCACACTGAGAA	90
			R: AAGGCAGGGCTCCCTAAGC	
Lactate dehydrogenase B	LDHB	NM_174100	F: CAGTCCTGCCTGCATCATCA	95
			R: TCACACGGTGCTTGGGTAATC	
Triosephosphate isomerase 1	TPI1	NM_001013589	F: GAGAAGGTCGTTTTCGAGCAA	100
			R: CAGTACCAATGGCCCACACA	
Tropomyosin 3	TPM3	NM_001011674	F: CTGAGAGATCGGTAGCCAAGCT	95
			R: CTCCTCGCTAATGGCCTTGT	
Enolase 3	ENO3	NM_001034702	F: CCCGACAAGGTGGTGATTG	95
			R: GCAGGGTCGTCAGGTGACTT	
Carbonic anhydrase III	CA3	NM_001034437	F: CACAGCGTGGATGGAGTCAA	100
			R: TACCATCGGCATGCTTCAGA	
Phosphoglucomutase 1	PGM1	NM_001076903	F: ACCCCAACTGGCTGGAAGTT	100
			R: CACGGATGTGGTCAGAACCA	
Lactate dehydrogenase A	LDHA	NM_174099	F: TCAGCTCGCTTCCGTTATCTC	85
			R: CACCATGCTCCCCAAGGAT	
Phosphoglycerate mutase 2	PGAM2	NM_001038111	F: ATCTGGAGGCGCTCCTTTG	80
			R: CGCTCCTTGCTGATGGACTT	
Phosphorylase, glycogen	PYGM	NM_175786	F: GGCCTGCTTTCTGGACTCAA	105
			R: TGCCAACCCCCAGAGATCT	
Pyruvate kinase	PKM	NM_001205727	F: CCTGCCTGCTGTGTCAGAAA	95
			R: AAGCCTTGCGGATGAAAGAC	
Malate dehydrogenase 1	MDH1	NM_001034628	F: TGGATGTGGCCATTCTTGTG	100
			R: GCACCCTGGCATTTGAAGAT	

PCR, polymerase chain reaction.

Table 2. Carcass characteristics among cows, steers, and bulls that were used in proteomic analysis

Variables	Cows (n = 7)	Steers (n = 7)	Bulls (n = 7)
Age (mo)	31.43±0.30	31.67±0.16	31.56±0.24
Carcass weight (kg)	406.10±13.37[b]	452.60±12.28[a]	490.90±13.59[a]
Backfat thickness (mm)	19.14±1.90[a]	16.67±1.51[a]	7.67±1.48[b]
Rib-eye area (cm^2)	90.57±2.45[b]	91.22±1.72[b]	100.90±3.46[a]
Yield index	61.47±1.47[b]	61.99±1.18[b]	67.95±0.97[a]
Yield grade[1]	142.90±20.20[b]	155.60±16.67[b]	277.78±14.70[a]
Marbling score[2]	4.71±0.47[b]	6.89±0.37[a]	1.00±0.00[c]
Quality grade[3]	32.86±1.84[b]	38.89±1.05[a]	10.00±0.00[c]

Mean±standard error of the mean.
[a-c] Means in row with different superscripts differ ($p<0.05$).
[1] Yield grade: 300 = A, 200 = B, 100 = C. [2] Marbling score: 1 = trace, 9 = very abundant. [3] Quality grade: 40 = 1++ or 1+, 30 = 1, 20 = 2, 10 = 3.

RESULTS AND DISCUSSION

Carcass characteristics

We used cows (636 kg live weight), steers (762 kg live weight), and bulls (832 kg live weight) at normal slaughter age (31 months) in Korea. Generally, Korean beefs are slaughtered routinely at 29 to 32 month of age to increase marbling and quality grade (Choy et al., 2012). Carcass characteristics of a subset data of the cows, steers, and bulls used for proteomic analysis are summarized in Table 2. Bulls had significantly ($p<0.05$) heavier carcass weight with lower trend backfat thickness. Bulls also had significantly ($p<0.05$) lower marbling scores, quality grade, and better yield grade. Our result was mostly in consistent with the effect of castration on meat quality in Korean cattle reported in a previous study (Jeong et al., 2013).

Protein profiles in IMAT and OMAT from Hanwoo cows, steers and bulls

To obtain a comprehensive overview of protein components in IMAT and OMAT from individual seven groups (cows, steers, and bulls), protein profiles of whole lysate of IMAT and OMAT were separated by SDS-PAGE and assessed by silver-stained image analysis. The number, marbling score, and quality grade of individuals were showed in Figure 1A. The patterns of total proteins in IMAT and OMAT were similar to each other. However, IMAT components were significantly different from OMAT components (Figure 1B).

Protein identification and gene ontological classification by LC-MS/MS-based proteomic analysis

LC-MS/MS-based proteomic analysis was performed to identify proteins involved in depot specific adipose tissue (*i.e.*

A

Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
ID number [a]	280449	280557	280433	280466	290224	290220	290199	292499	292441	282558	282661	282711	292334	282556	282559	282991	282998	283000	272558	292442	292440
Marbling score[b]	7	6	4	4	4	4	4	9	8	7	7	7	7	6	1	1	1	1	1	1	1
Quality grade[c]	1+	1+	1	1	1	1	1	1++	1++	1+	1+	1+	1+	1+	3	3	3	3	3	3	3

[a]ID number: Number of individuals. [b]Marbling score: 1= trace, 9 = very abundant. [c]Quality grade: 40 = 1++ or 1+, 30 = 1, 20 = 2, 10 = 3.

B

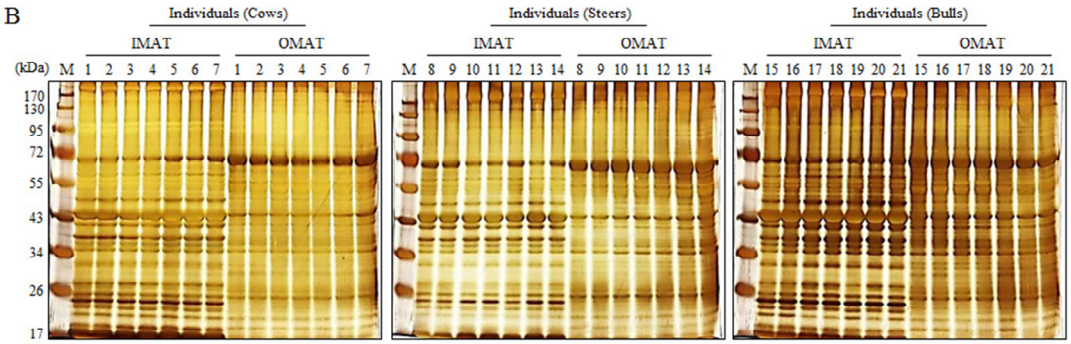

Figure 1. Protein profiles of intramuscular adipose tissue (IMAT) and omental adipose tissue (OMAT) from Hanwoo cows, steers and bulls by image analysis. (A) Number, marbling score, and quality grade of individuals; (B) Overall patterns of total protein bands from individuals (1 to 21). Gels were visualized by sliver staining.

intramuscular and omental) properties associated with sex (cows, steers, and bulls). Of the 55 proteins identified, 44, 40, and 42 proteins were confirmed to be differentially expressed in IMAT of cows, steers, and bulls, respectively. In OMAT of cows, steers, and bulls, 33, 33, and 22 were confirmed to be differentially expressed, respectively (Table 3). All identified proteins were clustered into eight categories based on biological process (BP) using information obtained from the DAVID gene ontology (GO) database (http://david.abcc.ncifcrf.gov) and UniProt (http://www.uniprot.org). Depending on the BP in which the proteins were involved, they were categorized into the following groups (Figure 2A): carbohydrate metabolism (35.7%), glycolysis (19.6%), muscle contraction (10.7%), electron transport (10.7%), protein folding (7.1%), muscle development (5.4%), tricarboxylic acid (TCA) pathway (5.4%), and carbon metabolism (5.4%).

A total of 16 up- or down-regulated proteins between

Table 3. List of total proteins in cows, steers and bulls among identified proteins between IMAT and OMAT

No	UniProt[1]	UniGene[2] (NCBI)	Protein identified	Gene name	pI[3]	MW (kDa)[4]	Seq. Cov (%)[5]	Individual ion score[6]					
								IMAT			OMAT		
								Cows	Steers	Bulls	Cows	Steers	Bulls
1	P02070	Bt.23726	Hemoglobin subunit beta	*HBB*	7.59	15.9	73.79	55.48	46.92	47.67	145.82	218.33	141.46
2	Q5KR48	Bt.53077	Tropomyosin beta chain	*TPM2*	4.7	32.8	62.68	90.86	84.76	146.88	6.92	5.24	0
3	P01966	Bt.10591	Hemoglobin subunit alpha	*HBA*	8.44	15.2	62.68	11.59	4.08	4.07	39.4	58.1	34.55
4	P68138	Bt.88733	Actin, alpha skeletal muscle	*ACTA1*	5.39	42	57.56	131.84	121.18	184.84	41.13	54.42	34.54
5	Q3T149	Bt.4415	Heat shock protein beta-1	*HSPB1*	6.4	22.4	56.72	24.45	10.58	46.52	46.03	45.34	32.87
6	A6QLL8	Bt.22533	Fructose-bisphosphate aldolase	*ALDOA*	8.19	39.4	43.41	60.43	58.92	88.1	21.42	12.71	8
7	Q9XSC6	Bt.3651	Creatine kinase M-type	*CKM*	7.12	43	39.9	40.7	55.66	111.25	0	0	0
8	Q5KR49	Bt.109484	Tropomyosin alpha-1 chain	*TPM1*	4.74	32.7	39.44	79.5	72.94	116.58	6.92	5.24	0
9	F1MHQ4	Bt.97	Fatty acid-binding protein, adipocyte	*FABP4*	5.66	14.6	39.39	0	5.73	0	35.8	33.58	25.77
10	P10096	Bt.87389	Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	8.35	35.8	39.04	31.57	41.42	78.81	4.86	5.48	5.5
11	P00171	Bt.65097	Cytochrome b5	*CYB5A*	5.03	15.3	32.09	4.3	0	0	6.18	0	0
12	Q5E9B1	Bt.7736	L-lactate dehydrogenase B chain	*LDHB*	6.44	36.7	31.44	0	0	3.78	29.61	27.85	13.08
13	Q5E956	Bt.3487	Triosephosphate isomerase	*TPI1*	6.92	26.7	31.33	9.57	20.36	30.91	2.97	3.06	0
14	Q5KR47	Bt.55987	Tropomyosin alpha-3 chain	*TPM3*	4.72	32.8	30.99	44.91	43.73	72.02	6.92	5.24	0
15	Q3ZC09	Bt.49475	Beta-enolase	*ENO3*	7.72	47.1	29.26	36.37	47.08	56.06	4.08	6.85	0
16	P02769	Bt.106669	Serum albumin	*ALB*	6.18	69.2	28.67	109.86	79.13	110.46	269.73	283.92	213.15
17	Q3SZX4	Bt.49056	Carbonic anhydrase 3	*CA3*	7.84	29.4	26.54	21.82	33.88	46.14	0	0	0
18	F1N647	Bt.30099	Fatty acid synthase	*FASN*	6.46	274.1	20.7	14.41	8.42	2.37	137.1	189.72	57.99
19	Q08DP0	Bt.59999	Phosphoglucomutase-1	*PGM1*	6.81	61.6	20.28	22.64	15.64	38.83	0	0	0
20	P19858	Bt.3809	L-lactate dehydrogenase A chain	*LDHA*	8	36.6	18.67	16.69	22.01	35.86	0	2.11	4.31
21	F1N2F2	Bt.23217	Phosphoglycerate mutase 2	*PGAM2*	8.9	28.7	16.21	8.57	3.86	19.32	0	0	0
22	P00829	Bt.4431	ATP synthase subunit beta, mitochondrial	*ATP5B*	5.27	56.2	15.34	11.48	4.89	11.38	19.53	17.88	10.08
23	F1MJ28	Bt.16003	Phosphorylase	*PYGM*	7.11	97.2	15.32	26.9	27.38	49.74	0	0	0
24	A4IFB3	Bt.24903	PLIN protein	*PLIN*	6.48	55	15.12	2.26	0	0	22.36	10.59	7.97
25	A5D984	Bt.40497	Pyruvate kinase	*PKM2*	7.85	57.9	14.69	25.77	20	35.69	0	0	0
26	Q3T0P6	Bt.37560	Phosphoglycerate kinase 1	*PGK1*	8.27	44.5	14.15	21.27	23.79	28.73	6.91	2.59	0
27	P00570	Bt.4224	Adenylate kinase isoenzyme 1	*AK1*	8.32	21.7	13.4	0	0	10.91	0	0	0
28	A7Z057	Bt.107001	14-3-3 protein gamma	*YWHAG*	4.89	28.3	13.36	5.63	6.21	4.93	15.39	18.16	8.32
29	A7E3W4	Bt.4750	Transketolase	*TKT*	7.14	64.8	11.58	4.49	0	0	26.36	29.15	11.84
30	Q27965	Bt.49659	Heat shock 70 kDa protein 1B	*HSPA1B*	5.92	70.2	11.08	10.32	7.47	18.99	16.86	17.4	7.77
31	F1MLB8	Bt.7194	ATP synthase subunit alpha	*ATP5A1*	9.19	59.7	9.22	14.33	6.72	12.58	8.05	10.82	6.99
32	Q3ZBY4	Bt.49614	Fructose-bisphosphate aldolase	*ALDOC*	6.65	39.4	9.07	18.16	17.87	24.52	6.22	4.62	0
33	Q3ZBD7	Bt.49587	Glucose-6-phosphate isomerase	*GPI*	7.71	62.8	8.62	7.58	2.57	9.5	3.34	0	0
34	Q3T100	Bt.1157	Microsomal glutathione S-transferase 3	*MGST3*	9.54	16.9	8.55	2.99	0	0	5.93	3.05	3.63
35	F1MB08	Bt.22783	Alpha-enolase	*ENO1*	6.8	47.3	8.53	18.17	10.23	15.29	4.08	6.85	0

Table 3. List of total proteins in cows, steers and bulls among identified proteins between IMAT and OMAT (Continued)

No	UniProt[1]	UniGene (NCBI)[2]	Protein identified	Gene name	pI[3]	MW (kDa)[4]	Seq. Cov (%)[5]	Individual ion score[6]					
								IMAT			OMAT		
								Cows	Steers	Bulls	Cows	Steers	Bulls
36	P42028	Bt.5483	NADH dehydrogenase	*NDUFS8*	6.87	23.9	8.02	2.09	4.12	0	0	0	0
37	P13696	Bt.59089	Phosphatidylethanolamine-binding protein 1	*PEBP1*	7.49	21	7.49	0	2.84	2.91	4.85	5.79	0
38	P19120	Bt.12309	Heat shock cognate 71 kDa protein	*HSPA8*	5.52	71.2	7.38	8.51	5.57	6.4	12.02	9.82	3.82
39	F1N7W0	Bt.15246	Uncharacterized protein	*MGC152281*	8.79	36.2	7.27	0	2.3	0	0	0	0
40	P15690	Bt.4777	NADH-ubiquinone oxidoreductase 75 kDa subunit	*NDUFS1*	6.15	79.4	6.74	0	2.57	0	0	0	0
41	P00432	Bt.48925	Catalase	*CAT*	7.28	59.9	6.45	2.12	0	0	8.86	9.6	10.08
42	Q8MKH7	Bt.11215	Troponin T fast skeletal muscle type	*TNNT3*	8.1	29.8	6	0	0	5.92	0	0	0
43	Q0V7M4	Bt.46979	Calcium-binding mitochondrial carrier protein SCaMC-2	*SLC25A25*	8.43	52.7	5.97	3.39	0	3.4	0	0	0
44	F1MGE7	Bt.62768	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	*ATP2A1*	5.27	109.2	5.74	8.57	10.49	12.02	0	0	0
45	Q08DM3	Bt.6984	Malic enzyme	*ME2*	7.65	65.4	5.31	0	0	2.37	0	0	0
46	Q1LZ96	Bt.59430	ATP synthase mitochondrial F1 complex assembly factor 2	*ATPAF2*	7.46	32.8	4.15	0	7.41	5.64	0	0	0
47	G1K1H1	Bt.7915	Malate dehydrogenase	*MDH2*	9.7	29.9	3.97	2.27	0	0	2.37	2.18	0
48	E1BLB2	Bt.29035	Uncharacterized protein	*TNFAIP1*	7.84	36.1	3.8	2.21	0	0	0	2.17	0
49	Q5E9C1	Bt.16018	Caspase-4	*CASP4*	6.18	43	3.71	2.23	2.25	4.29	0	0	0
50	Q29RL6	Bt.46395	Uncharacterized protein	*VAT1L*	5.1	45.8	3.58	3.77	0	0	0	0	0
51	Q8MKH6	Bt.4160	Troponin T, slow skeletal muscle	*TNNT1*	5.87	31.3	3.42	2.07	5.48	3.78	0	0	0
52	P02453	Bt.23316	Collagen alpha-1(I) chain	*COL1A1*	5.78	138.9	3.42	6.11	4.35	8.69	0	3.35	2.86
53	F1MYC8	Bt.3961	Calpain-3	*CAPN3*	6.29	82.6	3.39	4.6	0	0	2.36	4.66	4.79
54	Q3T145	Bt.5345	Malate dehydrogenase, cytoplasmic	*MDH1*	6.58	36.4	2.99	0	0	4.41	0	0	0
55	G5E6M7	Bt.24449	Succinate dehydrogenase	*SDHA*	7.62	73.2	2.11	2.92	0	0	3.01	3.19	0

IMAT, intramuscular adipose tissue; OMAT, omental adipose tissue.
[1] UniProt, accession number in the UniProt database. [2] UniGene: UniGene number from NCBI (National Center for Biotechnology Information) database.
[3] pI, isoelectric point of the protein. [4] MW (kDa), molecular weight of the protein. [5] Seq. Cov (%), percentage of sequence coverage.
[6] Individual ion score, TurboSEQUEST or gMASCOT score.

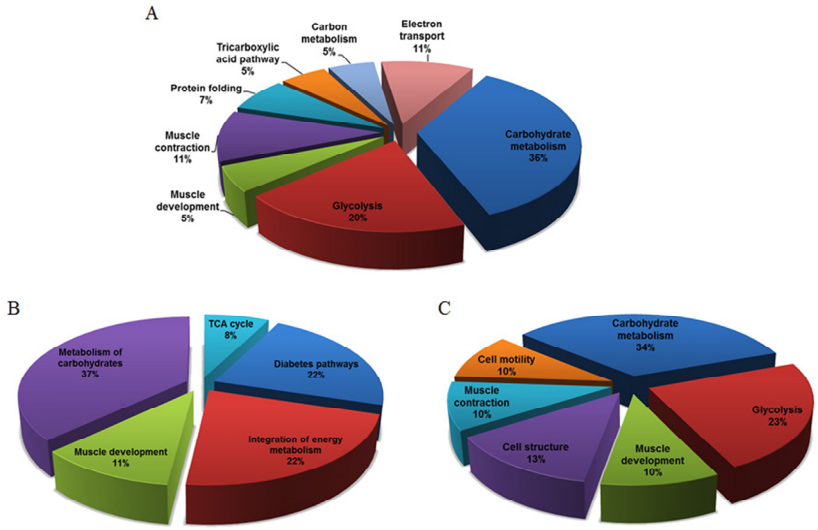

Figure 2. Ontological classification of differentially regulated proteins in intramuscular adipose tissue (IMAT) and omental adipose tissue (OMAT) from Hanwoo cows, steers and bulls. Of the 55 identified proteins, 44, 40, and 42 proteins were differentially expressed in IMAT of cows, steers, and bulls, respectively. In OMAT, 33, 33, and 22 were differentially expressed in cows, steers, and bulls, respectively (A) Identified proteins were clustered into eight categories based on their biological processes. Representative category of the 16 up- or down-regulated proteins between IMAT and OMAT of cows, steers, and bulls; (B) Depending on the reactome-pathway, the proteins were clustered into five categories; (C) Depending on the panther-biological processes, the proteins were clustered into six categories.

IMAT and OMAT of cows, steers, and bulls were selected. GO analysis was performed using DAVID Bioinformatics Resources 6.7 categories both Reactome-Pathway and Panther-BP. Depending on the Reactome-Pathway in which the protein was involved, the 16 proteins were categorized into the following five groups (Figure 2B): metabolism of carbohydrates (37%), integration of energy metabolism (22.2%), diabetes pathways (22.2%), muscle development (11.1%), and TCA cycle (7.4%). Depending on the Panther-BP in which the protein was involved, they were categorized into the following six groups (Figure 2C): carbohydrate metabolism (33.3%), glycolysis (23.3%), cell structure (13.3%), muscle development (10%), muscle contraction (10%), and cell motility (10%) (Table 4). The expression changes of the up- and down-regulated proteins in IMAT and OMAT of cows, steers, and bulls depending on the Reactome-Pathway were summarized in Table 4. The mRNA expression patterns of the 16 selected proteins were further analyzed by real-time PCR.

Quantitative real-time PCR confirmation for selected genes

To study the patterns of gene expression in IMAT and OMAT associated with sex, we used cows, steers, and bulls. The mRNA expression levels of the selected genes were subjected to quantitative real-time PCR with specific primers (Table 1). Previous studies have reported that fructose-bisphosphate aldolase A (ALDOA) mRNA increases during *in vitro* myogenesis (Colbert and Ciejek-Baez, 1988) are responsible for significant activation during the differentiation of primary myoblasts, therefore playing important roles in muscle gene transcription (Walsh et al., 1980; Hidaka et al., 1993; Ren et al., 2011). Our data showed that ALDOA had significantly higher expression in IMAT than in OMAT in cows ($p = 0.0042$) and steers ($p<0.0001$) (Figure 3A and 3B). However, ALDOA had significantly ($p<0.0001$) lower expression in IMAT than in OMAT in bulls (Figure 3C). These results demonstrated that ALDOA was differentially expressed depending on sex, suggesting that ALDOA could be one of the factors affecting lipid accumulation in OMAT.

Western blot analysis for selected proteins

We found significant correlations between several factors (including tropomyosin [TPM] 1, TMP2, and TMP3) and gene expression in IMAT and OMAT. TPMs are a family of actin binding proteins in all tissues that are always associated with polymerized actin. TPMs are a diverse group of cytoskeletal proteins found in most eukaryotic cells, with distinct isoforms found in muscle (skeletal, cardiac, and smooth) and various non-muscle cells (Dlugosz et al., 1984; Lin and Lin, 1986). Previous studies have shown that TPM

Table 4. Reactome pathway related proteins in cows, steers and bulls among identified proteins between IMAT and OMAT

No	UniProt[1]	UniGene[2] (NCBI)	Protein identified	Gene name	pI[3]	MW (kDa)[4]	Seq. Cov (%)[5]	Individual ion score[6]					
								IMAT			OMAT		
								Cows	Steers	Bulls	Cows	Steers	Bulls
Metabolism of carbohydrates													
1	Q3SZX4	Bt.49056	Carbonic anhydrase 3	*CA3*	7.84	29.4	26.54	21.82	33.88	46.14	0	0	0
2	Q08DP0	Bt.59999	Phosphoglucomutase-1	*PGM1*	6.81	61.6	20.28	22.64	15.64	38.83	0	0	0
3	F1MJ28	Bt.16003	Phosphorylase	*PYGM*	7.11	97.2	15.32	26.9	27.38	49.74	0	0	0
4	Q3T145	Bt.5345	Malate dehydrogenase, cytoplasmic	*MDH1*	6.58	36.4	2.99	0	0	4.41	0	0	0
Integration of energy metabolism/Diabetes pathways													
5	A6QLL8	Bt.22533	Fructose-bisphosphate aldolase A	*ALDOA*	8.19	39.4	43.41	60.43	58.92	88.1	21.42	12.71	8
6	P10096	Bt.87389	Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	8.35	35.8	39.04	31.57	41.42	78.81	4.86	5.48	5.5
7	Q5E956	Bt.3487	Triosephosphate isomerase	*TPI1*	6.92	26.7	31.33	9.57	20.36	30.91	2.97	3.06	0
8	Q3ZC09	Bt.49475	Beta-enolase	*ENO3*	7.72	47.1	29.26	36.37	47.08	56.06	4.08	6.85	0
9	F1N2F2	Bt.23217	Phosphoglycerate mutase 2	*PGAM2*	8.9	28.7	16.21	8.57	3.86	19.32	0	0	0
10	A5D984	Bt.40497	Pyruvate kinase	*PKM2*	7.85	57.9	14.69	25.77	20	35.69	0	0	0
Muscle development													
11	Q5KR48	Bt.53077	Tropomyosin beta chain	*TPM2*	4.7	32.8	62.68	90.86	84.76	146.88	6.92	5.24	0
12	P68138	Bt.88733	Actin, alpha skeletal muscle	*ACTA1*	5.39	42	57.56	131.84	121.18	184.84	41.13	54.42	34.54
13	Q5KR49	Bt.109484	Tropomyosin alpha-1 chain	*TPM1*	4.74	32.7	39.44	79.5	72.94	116.58	6.92	5.24	0
14	Q5KR47	Bt.55987	Tropomyosin alpha-3 chain	*TPM3*	4.72	32.8	30.99	44.91	43.73	72.02	6.92	5.24	0
TCA cycle													
15	Q5E9B1	Bt.7736	L-lactate dehydrogenase B chain	*LDHB*	6.44	36.7	31.44	0	0	3.78	29.61	27.85	13.08
16	P19858	Bt.3809	L-lactate dehydrogenase A chain	*LDHA*	8	36.6	18.67	16.69	22.01	35.86	0	2.11	4.31

IMAT, intramuscular adipose tissue; OMAT, omental adipose tissue; TCA, tricarboxylic acid.
[1] UniProt, Accession number in the UniProt database. [2] UniGene, UniGene number from NCBI (National Center for Biotechnology Information) database.
[3] pI, isoelectric point of the protein. [4] MW (kDa), molecular weight of the protein. [5] Seq. Cov (%), percentage of sequence coverage.
[6] Individual ion score, TurboSEQUEST or gMASCOT score.

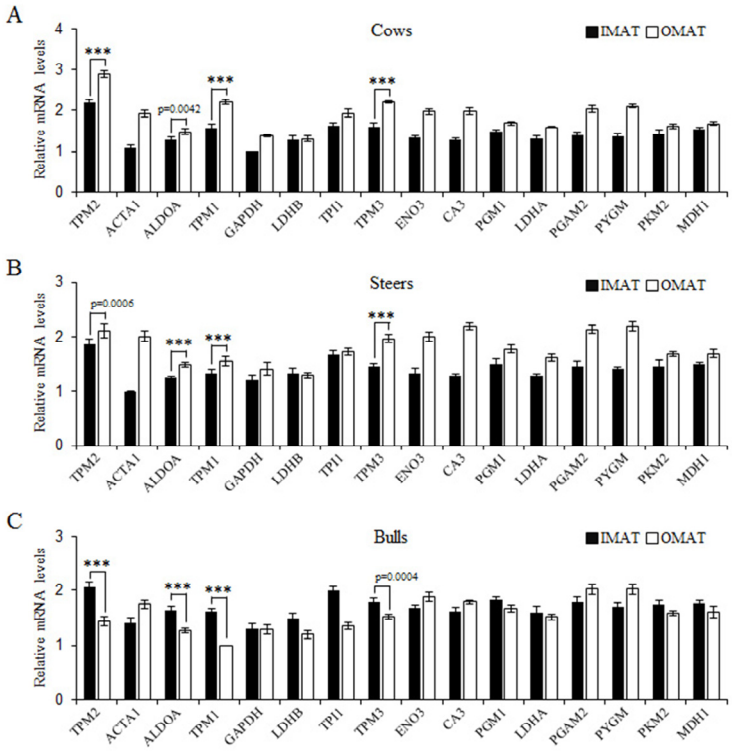

Figure 3. Gene expression levels on intramuscular adipose tissue (IMAT) and omental adipose tissue (OMAT) depending on sex. The quantitative differences of 16 genes at the transcriptional level were measured by real-time polymerase chain reaction in IMAT and OMAT from Hanwoo cows, steers, and bulls. TPM2, tropomyosin 2; ACTA1, actin, alpha 1, skeletal muscle; ALDOA, fructose-bisphosphate aldolase A; TPM1, tropomyosin 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDHB, lactate dehydrogenase B; TPI1, triosephosphate isomerase 1; TPM3, tropomyosin 3; ENO3, enolase 3; CA3, carbonic anhydrase III; PGM1, phosphoglucomutase 1; LDHA, lactate dehydrogenase A; PGAM2, phosphoglycerate mutase 2; PYGM, phosphorylase, glycogen; PKM, pyruvate kinase; MDH1, malate dehydrogenase 1. Student's t test was performed to evaluate statistical significance (*** $p<0.0001$; mean±standard error of the mean; n = 3).

plays a critical role in skeletal muscle development and function (Marston et al., 2013; Zhang et al., 2014). Results of the mRNA levels (upper panels) and protein expression levels (lower panels) of TPM1, TPM2, and TPM3 are shown in Figure 4. Notably, transcriptional and protein levels of TPM1, TPM2, and TPM3 were significantly lower in IMAT

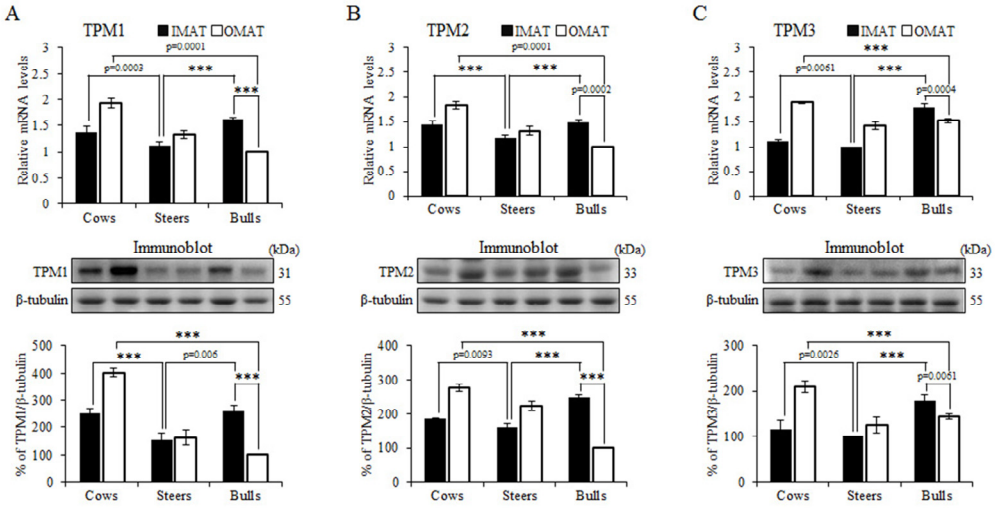

Figure 4. Gene and protein expression levels of tropomyosin (TPM)1, TPM2, and TPM3 in intramuscular adipose tissue (IMAT) and omental adipose tissue (OMAT). The quantitative differences of (A) TPM1, (B) TPM2, and (C) TPM3 at the transcriptional and protein levels were measured by real-time polymerase chain reaction and western blot analysis. Student's t test was performed to evaluate statistical significance (*** $p<0.0001$; mean±standard error of the mean; n = 3).

of steers compared to cows or bulls. The mRNA and protein levels of TPM1, TPM2, and TPM3 were higher in OMAT of cows than in bulls. In addition, TPM1, TPM2, and TPM3 had higher expression in OMAT than in IMAT in cows and steers, but had lower expression in OMAT than IMAT in bulls. These results demonstrated that TPM1, TPM2, and TPM3 were differentially expressed depending on sex. Adipose depots and TPMs were positively correlated with marbling score and quality grade. Therefore, we suggest that TPM1, TPM2, and TPM3 are key factors closely associated with muscle development and lipid accumulation in Hanwoo cows, steers, and bulls.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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