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
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., 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 Steinhardt, 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 PROPREP protein extraction solution (iNIRON 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 sensitzation 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^{ΔΔCt}) 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 R: GCC GAG CTA CTT CAT TGT GG | 211               |
| Actin, alpha 1, skeletal muscle           | ACTA1  | NM_174225    | F: GAGCCGTGCTACTCTCCTGTT R: GGCTGCAATTTCCGTCTGCT | 105               |
| Aldolase A, fructose-bisphosphate         | ALDOA  | NM_001101915 | F: CCAGCCCTGACTCCAGAAAT R: CTCGCGAGAAGGATGATC | 110               |
| Tropomyosin 1                            | TPM1   | NM_001013590 | F: GGTGCGACAGAAAGGATGAT R: GCACATTTGCTCCTGCAAAGG | 105               |
| Glyceraldehyde-3-phosphate dehydrogenase | GAPDH  | NM_001034034 | F: CATCTCCGCCACACTGAGAA R: AAGGCAGGGCTCCTCAGA | 90                |
| Lactate dehydrogenase B                  | LDHB   | NM_174100    | F: CAGTCTCCCTGACTGATCA R: TCAACAGCGCTGCTGGGTAATC | 95                |
| Triosephosphate isomerase 1              | TPI1   | NM_001013589 | F: GAGAGGCTGTTTCTGGAACAA R: CAGTACAAATGCCACACAAC | 100               |
| Tropomyosin 3                            | TPM3   | NM_001011674 | F: CTGAGGAGATGCTGGGACTCA R: CTCGCGCTAATCGTGGTTG | 95                |
| Enolase 3                                | ENO3   | NM_001034702 | F: CCCCCACAGGTGTTGATTTG R: GCAGGCGCTCAGTTGACCTT | 95                |
| Carbonic anhydrase III                   | CA3    | NM_001034437 | F: CACAGGCGATGAGTTGCAA R: TACCATCGCATGCTTCAAG | 100               |
| Phosphoglucomutase 1                     | PGM1   | NM_001076903 | F: ACCCCAATCTGCTGGAATT R: CACGGAGTGTGCTGGGACAAC | 100               |
| Lactate dehydrogenase A                  | LDHA   | NM_174099    | F: TCAGTCGCTTCCGTATCTC R: CAGTGCTCCACAGAGGATCT | 85                |
| Phosphoglycerate mutase 2                | PGAM2  | NM_001038111 | F: ATCTGGAGCGCTCCCTTGG R: CGCTGCTGGTAGATGACTC | 80                |
| Phosphorylase, glycogen                  | PYGM   | NM_175786    | F: GCCGCTGGTCTGACTC R: TGCCACCCCGACAGAC | 105               |
| Pyruvate kinase                          | PKM    | NM_001205727 | F: CTCGCTGCTGCTGCTGACTC R: AGGAAAAAACCATTCAGAGAC | 95                |
| Malate dehydrogenase 1                   | MDH1   | NM_001034628 | F: TGGATGAGGCTTCTGTTG R: GCACCTGGCCATTGGAAGAT | 100               |

PCR, polymerase chain reaction.
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).

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±13.37b| 452.60±12.28a | 490.90±13.59a|
| Backfat thickness (mm)| 19.14±1.90a  | 16.67±1.51a     | 7.67±1.48b   |
| Rib-eye area (cm²) | 90.57±2.45b  | 91.22±1.72b    | 100.90±3.46a |
| Yield index        | 61.47±1.47b  | 61.99±1.18b    | 67.95±0.97a  |
| Yield grade¹       | 142.90±20.20b| 155.60±16.67b  | 277.78±14.70a|
| Marbling score²    | 4.71±0.47b   | 6.89±0.37a     | 1.00±0.00c   |
| Quality grade³     | 32.86±1.84b  | 38.89±1.05a    | 10.00±0.00c  |

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.

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.
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 IMAT and OMAT were identified (Table 3). The proteins were involved in various biological processes, including carbohydrate metabolism, glycolysis, muscle contraction, electron transport, protein folding, muscle development, the TCA pathway, and carbon metabolism. The list of proteins included Hemoglobin subunit beta (HBB), Tropomyosin beta chain (TPM2), Hemoglobin subunit alpha (HBA), Actin, alpha skeletal muscle (ACTA1), Heat shock protein beta-1 (HSPB1), Fructose-bisphosphate aldolase (ALDOA), Creatine kinase M-type (CKM), Tropomyosin alpha-1 chain (TPM1), Fatty acid-binding protein, adipocyte (FABP4), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Cytochrome b5 (CYB5A), L-lactate dehydrogenase B chain (LDHB), Triosephosphate isomerase (TPI1), Tropomyosin alpha-3 chain (TPM3), Beta-enolase (ENO3), L-lactate dehydrogenase A chain (LDHA), L-lactate dehydrogenase B chain (LDHB), Phosphoglycerate mutase 2 (PGAM2), ATP synthase subunit beta, mitochondrial (ATPSB), Phosphorylase (PYGM), PLIN protein (PLIN), Pyruvate kinase (PKM2), Phosphoglycerate kinase 1 (PGK1), Adenylate kinase isoenzyme 1 (AK1), Microsomal glutathione S-transferase 3 (MGST3), and Alpha-enolase (ENO1).
Table 3. List of total proteins in cows, steers and bulls among identified proteins between IMAT and OMAT (Continued)

| No | UniProt ID | UniGene (NCBI) | Protein identified | Gene name | pI | MW (kDa) | Seq. Cov (%) | Individual ion score |
|----|------------|----------------|-------------------|-----------|----|---------|--------------|---------------------|
| 36 | P42028     | Bt.5483        | NADH dehydrogenase | NDUF58    | 6.87 | 23.9 | 8.02 | 2.09; 4.12 |                      |
| 37 | P13696     | Bt.59089       | Phosphatidylethanolamine-binding protein 1 | PEBP1    | 7.49 | 21 | 7.49 | 0; 2.84; 2.91 | 4.85; 5.79 | 0; 0 |
| 38 | P19120     | Bt.12309       | Heat shock cognate 71 kDa protein | HSP48    | 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 |
| 40 | P15690     | Bt.4777        | NADH-ubiquinone oxidoreductase 75 kDa subunit | NDUF51   | 6.15 | 79.4 | 6.74 | 0; 2.57 | 0; 0; 0 |
| 41 | P00432     | Bt.48925       | Catalase | CAT      | 7.28 | 59.9 | 6.45 | 2.12 | 0; 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; 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       | Sarcomplasmic/endoplasmic reticulum calcium ATPase | 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; 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; 2.37 | 2.18 | 0 |
| 48 | E1BLB2     | Bt.29035       | Uncharacterized protein | TNAIP1   | 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; 0 |
| 51 | Q8MKH6     | Bt.4160        | Troponin T, slow skeletal muscle type | 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 | 3.92 | 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; 2.36; 4.66; 4.79 |
| 54 | Q5T145     | Bt.5345        | Malate dehydrogenase, cytoplasmic | MDH1     | 6.58 | 36.4 | 2.99 | 0; 4.41 | 0; 0; 0 |
| 55 | G5E6M7     | Bt.24449       | Succinate dehydrogenase | SDHA     | 7.62 | 73.2 | 2.11 | 2.92 | 0; 3.01; 3.19 | 0; 0; 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 analyzed by real-time PCR.

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

| No | UniProt¹ | UniGene² (NCBI) | Protein identified | Gene name | pI³ | MW (kDa)⁴ | Seq. Cov. (%)⁵ | Individual ion score⁶ | IMAT | OMAT |
|----|----------|-----------------|--------------------|-----------|-----|---------|------------|-------------------|------|------|
|    |          |                 |                    |           |     |         |            |                   | Cows | Steers | Bulls | Cows | Steers | Bulls |
| 1  | Q3SZX4   | Bt.49056        | Carbonic anhydrase 3 | CA3       | 7.84| 29.4    | 26.54      | 21.82             | 33.88| 46.14 | 0     | 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      | 0     |
| 3  | F1MJ28   | Bt.16003        | Phosphorylase       | PYSM      | 7.11| 97.2    | 15.32      | 26.9              | 27.38| 49.74 | 0     | 0    | 0      | 0     |
| 4  | Q3T145   | Bt.5345         | Malate dehydrogenase, cytoplasmic | MDH1 | 6.58| 36.4   | 2.99       | 0                | 4.41 | 0     | 0     | 0    | 0      | 0     |

**Metabolism of carbohydrates**

1. Fructose-bisphosphate aldolase A (ALDOA) mRNA increases during *in vitro* myogenesis (Colbert and Ciejek-Baez, 1988) and plays an important role 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...
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
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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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