JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Heat Shock Protein B1 and Its Regulator Genes Are Negatively Correlated with Intramuscular Fat Content in the Longissimus Thoracis Muscle of Hanwoo (Korean Cattle) Steers

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ABSTRACT: In previous proteomic studies, heat shock protein β 1 (HSPB1) was detected as a candidate protein related to meat quality in cattle. This study sought to determine if its gene expression was associated with intramuscular fat content in the longissimus thoracis muscle of Korean cattle (Hanwoo). Tissue from two groups of 10 steers each, low-marbling (mean intramuscular fat content, 7.4 ± 1.5%) and high-marbling (23.5 ± 2.8%), were used for immunoblotting, real-time PCR, and statistical analyses. HSPB1 expression in both mRNA and protein was shown to be negatively related to intramuscular fat content ($P < 0.05$). Pathway analysis found two genes, TNF receptor superfamily member 6 (FAS) and angiotensinogen (AGT), that were regulators of the HSPB1 gene. The expression of the two genes showed a negative correlation with intramuscular fat content ($P < 0.05$). These results suggest that HSPB1, FAS, and AGT may be good candidate genes associated with intramuscular fat content in the longissimus muscle of Korean cattle.

KEYWORDS: HSPB1, FAS, AGT, intramuscular fat, Korean cattle

INTRODUCTION

In the Korean beef industry, the tenderness, flavor, and marbling (intramuscular fat) of meats are very important factors in the determination of economic value. Among these factors, marbling is particularly coveted by Korean consumers and producers. Therefore, identification of genes regulating marbling phenotype (IMF) will be useful for more targeted meat production in the livestock industry.

Marbling is a quantitative trait that is controlled by multiple genes and numerous environmental effects. Transcriptomic and proteomic studies have attempted to identify genes affecting phenotypic differences for marbling and tenderness in cattle using high-density microarrays and two-dimensional electrophoresis.[1] Other more specific studies that selected candidates according to these molecular functions provided a better understanding of muscle physiological processes and their influence on meat quality.[2,3] These studies demonstrated that expression analysis is very important for understanding the molecular mechanisms associated with phenotypic characteristics in animals.

Previously, we identified heat shock protein β 1 (HSPB1) as a candidate in m. longissimus muscle through a proteomic study.[4] However, the relationship between the expression of HSPB1 and intramuscular fat content was not clear. In the present study, we determined whether HSPB1 was associated with intramuscular fat content using immunoblotting, real-time PCR, and statistical analyses of low- and high-marbled m. longissimus muscle samples. We also performed pathway analysis to identify regulator genes related to HSPB1 expression and intramuscular fat content.

MATERIALS AND METHODS

Animals and Sample Preparation. Twenty samples of low- and high-marbled m. longissimus from Hanwoo (n = 10 animals in each group) steers were obtained from the junction between the 11th and 12th lumbar vertebrae within 30 min of slaughter. The selected tissues were placed in liquid nitrogen, ground to a fine powder using a mortar, and stored at −80 °C. All experimental procedures and care of animals were conducted in accordance with the guidelines of the Animal Care and Use Committee of the National Institute of Animal Science in Korea.

Measurement of Meat Quality. The fat content in the m. longissimus muscle samples was analyzed using the methods of the Association of Official Analytical Chemists (AOAC).[5] Objective meat color (CIE L, a, b) was determined using a Minolta Chromameter CR300 (Minolta, Japan) on freshly cut surfaces of meat after 30 min of blooming at 1 °C. Warner−Bratzler (WB) shear force (kg) was measured on cooked steaks (2.54 cm thick) that were placed in a preheated water bath for 60 min or until the core temperature reached 70 °C and subsequently cooled in running water (ca. 18 °C) for 30 min to reach a core temperature of <30 °C. Eight 1.27 cm diameter cores were obtained from each sample, and peak force was determined using a V-shaped shear blade with a cross-head speed of 400 mm/min.[6] For identifying the characteristic differences of meat quality traits between low- and high-marbled samples, statistical analysis was performed by an analysis of

Received: January 17, 2011
Revised: April 14, 2011
Accepted: April 14, 2011
Published: April 27, 2011

Table 1. Primer Sequences for Real-Time PCR

gene symbol	full name		primer sequence (5′−3′)	Genbank accession no.
HSPB1	heat shock protein beta 1	forward	AGATCACTGGCAAGCACGAGGAAA	NM_001025569
		reverse	GGGCAGCGTGTATTTGCGAGTGAA	
FAS	TNF receptor superfamily, member 6	forward	GGGAGATGAATGCTGGTGAGAGTA	NM_174662.2
		reverse	CAACCTGTGGATAGGCATGTGTGT	
F2	coagulation factor II	forward	TGAGGACCATTTCCAGCCCTTCTT	NM_173877.1
		reverse	AAAGCTCCTTCTCCGTTTGGTCCT	
TGFB1	transforming growth factor, β 1	forward	TAGCAACAATTCCTGGCGCTACCT	NM_001166068.1
		reverse	TGAGGCGAAAGCCCTCTATTTCCT	
FGF2	fibroblast growth factor 2	forward	TCGCTAAGAGTTGGACACGACTGA	NM_174056.3
		reverse	GTGGGTTGCCATTTCCTTCTCCAA	
EDN1	endothelin 1	forward	CCCAGTGACCTGCAAATTGGGATT	NM_181010.2
		reverse	ACAAACACTCCTTCGGACCCTCTT	
AGT	angiotensinogen	forward	TCGCTGCTGAGAAGATCAACAGGT	NM_001114082.1
		reverse	AGTGGACGTAGGCATTGAAGAGCA	
IFNG	interferon, gamma	forward	GCAGATCCAGCGCAAAGCCATAAA	NM_174086.1
		reverse	AGGCAGGAGGACCATTACGTTGAT	
ESR1	estrogen receptor 1	forward	GAATGTTGAAGCACAAGCGCCAGA	NM_001001443.1
		reverse	ACCGGGCTGTTCTTCTTAGTGTGT	
STAT3	signal transducer and activator of transcription 3	forward	CACCAAGCAGCAGCTGAACAACAT	NM_001012671.2
		reverse	TACTTTCCGAACGCGTCCTCCTTT	
HSP90AA1	heat shock 90kD protein 1, α	forward	TTAGTGTCAGTCACCAAAGAGGGC	NM_001012670.1
		reverse	CAACCGGTTTGACACAACCACCTT	
STAT1	signal transducer and activator of transcription 1	forward	ATTGGTTCACCATAGTGGCGGAGA	AY488117.1
		reverse	ATGAGCTCTGAATGAGCTGCTGGA	
ATF5	similar to transcription factor-like protein ODA-10	forward	ATGACCGAACGGGTGGACTTTACA	XM_593712.4
		reverse	TTCCATCTGCTCCAGCTCCTTCTT	
PSIP1	PC4 and SFRS1 interacting protein	forward	AAACTGAGGAGGCTGGAGTAGTGA	XM_590212.4
		reverse	TTGGAATCTTGACCTCTGTGGCTG	
ATF3	activating transcription factor 3	forward	TCGCACCATTAGGATCCATGCAGA	NM_001046193.1
		reverse	TTTGCTAGAAAGCACAACCCTGGC	
HSF1	heat shock transcription factor 1	forward	CCCAGCAACAGAAAGTCGTCAACA	NM_001076809.1
		reverse	CCGTGGATATGCTCCAGCGAGTA	
POU4F2	brn3b POU domain transcription factor, transcript variant 2	forward	TGAGCACAACTTTGCATCGCCCTT	XM_864175.2
		reverse	CCTGCTTGCTGTTCAAGGACATCATC	
NR1H4	nuclear receptor subfamily 1, group H, member 4	forward	ATCTGGGATGTGCAGTGATGAGCA	NM_001034708.1
		reverse	TGGCATGAAGCCAGGTACAAATGC	

Table 1. Continued

gene symbol	full name		primer sequence (5′−3′)	Genbank accession no.
SP1	Sp1 transcription factor	forward	CGTCTTGCCAACAGTGGCATCAAT	NM_001078027.1
		reverse	AGGTGCCTGATCTCAGAAGCCATT	
POU4F1	POU class 4 homeobox 1	forward	TCTCACGCTCTCGCACAACAACAT	XM_001253067.1
		reverse	ATGGAAGTCCGCTTGCGCTTCTT	
HSF2	heat shock transcription factor 2	forward	TGGGAACCCTGCTTCTTCTGTTGA	NM_001083405.1
		reverse	TTGGTTCTGGGTCCAAGCTGCTAT	
C/EBPα	CCAAT/enhancer binding protein, α	forward	AGAAGTCCGTGGACAAGAACAGCA	NM_176784.2
		reverse	ATTGTCACTGGTCAGCTCCAGCA	
PPARγ	peroxisome proliferator-activated receptor γ	forward	AGCCTCATGAAGAGCCTTCCAACT	NM_181024.2
		reverse	TCCATAGTGGAACCCTGACGCTTT	
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	forward	GGGTCATCATCTCTGCACCT	BC102589
		reverse	GGTCATAAGTCCCTCCACGA	
β-actin	actin, β	forward	ACCTGCGAAGATGTGTCTGAACCA	NM_173979.3
		reverse	TGTACACCGTCAGCTTGTTGAGGA	
RPLP0	ribosomal protein, large, P0	forward	TCTTCCAGGCTTTAGGCATCACCA	NM_001012682.1
		reverse	GATGTTCAGCATGTTCAGCAGCGT	
18S rRNA	18S rRNA	forward	GTAACCCGTTGAACCCCATT	DQ222453.1
		reverse	CCATCCAATCGGTAGTAGCG	

variance (ANOVA) model using the MIXED procedure in an R statistical package for animal nested within age as the random effect.

Quantitative Real-Time PCR. Total RNA was prepared from each tissue sample (100 mg) using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and then purified using an RNeasy MinElute Clean-up kit (Qiagen, Valencia, CA). RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA), which indicated that the RNA purity (A_{260}/A_{280}) was >1.90. For cDNA synthesis, 2 μg of RNA was reverse transcribed in a 20 μL reaction volume using random primers (Promega, Madison, WI) and reverse transcriptase (SuperScript II Reverse Transcriptase, Invitrogen Life Technologies). Reactions were incubated at 65 °C for 5 min, at 42 °C for 50 min, and then at 70 °C for 15 min to inactivate the reverse transcriptase.

Real-time PCR was performed using the 2X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with the 7500 Real Time PCR system (Applied Biosystems) using 10 pM of each primer (Table 1). The PCR was run for 2 min at 50 °C and for 10 min at 95 °C, followed by 40 cycles of 95 °C for 10 s and then 60 °C for 1 min. Following amplification, a melting curve analysis was performed to verify the specificity of the reaction. The end point used in the real-time PCR quantification, Ct, was defined as the PCR threshold cycle number.

For selection and use of internal control genes, we calculated gene-stability (*M*) values and identified two genes among the commonly used housekeeping (HK) genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, ribosomal protein, large, P0 (RPLP0), and 18S rRNA (18S rRNA) using the *geNorm* analysis. The RPLP0 gene showed the lowest *M* value, and the best two-gene combination was RPLP0 and β-actin, the *M* value of which was 0.393; this value met the stability requirement to be an HK gene. Therefore, the RPLP0 and β-actin genes were selected as the internal controls for normalization by geometric mean of the two selected HK genes.[7]

Immunoblotting. For protein extraction from individual samples, frozen muscle tissue (100 mg) was incubated for 40 min in 1 mL of 8 M urea, 2 M thiourea, 65 mM dithiothreitol (DTT), and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Samples were centrifuged at 40000*g* for 30 min, and the supernatants were used as the protein extracts. Protein concentration was determined using the protein assay system (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as a standard. For immunoblotting, 30 μg of sample proteins was separated on SDS-PAGE according to the Laemmli method,[8] and gels were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) in ice-cold transfer buffer (25 mM Tris-Cl, pH 8.3, 1.4% glycine, 20% methanol) at 250 mA for 60 min. Membranes were treated with blocking buffer containing 3% nonfat milk (Becton, Dickinson and Co., Sparks, MD) in TBS/T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) and were incubated overnight at 4 °C. Primary goat antibodies (sc-1048, Santa Cruz, Biotechnology, Santa Cruz, CA) against HSP27 encoded by HSPB1 were used at 1:200 dilutions in TBS/T with 3% nonfat milk. Following 2 h of incubation with the primary antibodies, membranes were washed three times for 10 min each with 10 mL of TBS/T. Horseradish peroxidase-labeled (HRP) anti-goat secondary antibody was diluted 1:1000 in TBS/T with 3% nonfat milk and incubated for 2 h. After three 10 min washes, membranes were visualized using a chemiluminescent HRP substrate (Millipore) and a VersaDoc image analyzer (Bio-Rad). The band densities were calculated using Quantity One software (Bio-Rad, ver. 3.1) and normalized according to the density of α-tubulin (sc-12462, Santa Cruz Biotechnology, Inc.).

Table 2. Characteristics of Meat Quality Traits between Low- and High-Marbled Groups (Mean ± SD)[a]

		CIE					
group	age (months)	*L*	*a*	*b*	CW (kg)	IMF (%)	WBS (kg)
low	27.1 ± 0.9	35.1 ± 1.7	21.2 ± 3.4	9.7 ± 2.2	409.2 ± 33.5	7.4 ± 1.5	5.1 ± 1.1
high	29.5 ± 1.5	37.5 ± 2.0**	21.5 ± 1.1	9.6 ± 0.7	478.2 ± 43.1	23.5 ± 2.8***	3.5 ± 1.0*

[a] CW, carcass weight; IMF, intramuscular fat content; WBS, Warner−Bratzler shear force. *, **, ***: significant differences ($P < 0.05$, 0.01, and 0.001, respectively) between low- and high-marbled groups determined using a linear mixed model to fit animal nested within age as random effect.

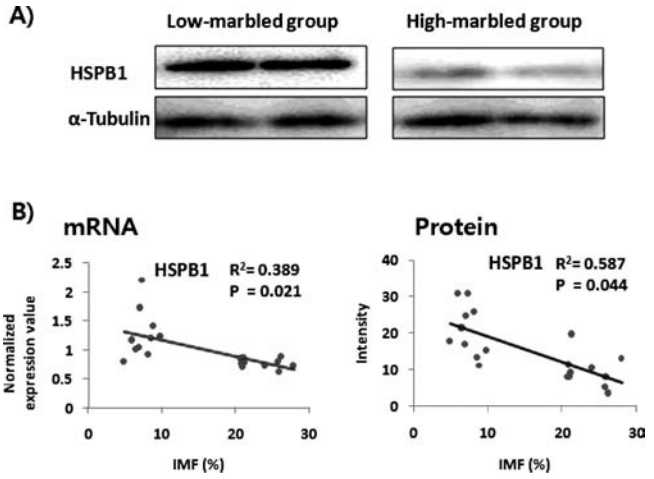

Figure 1. Expression profiles of HSPB1 in the longissmus tissue of Korean cattle (Hanwoo) steers: (A) result of Western blotting of HSPB1 protein between low- and high-marbled groups; (B) regression analysis between normalized expression level (*y*-axis) from real-time PCR (mRNA) and Western blotting (protein) and intramuscular fat content (%) for each sample.

Pathway Analysis. The Pathway Studio (version 6.0, Stratagene, La Jolla, CA) program was used to identify upstream proteins associated with the HSPB1 gene. This software uses a proprietary database that references protein interaction data generated from PubMed to obtain a biological association network (BAN) of known protein interactions. After experimental validation, we also examined the relationships and the effects of regulations (positive, negative, and unknown) among the significant genes in our analysis.

Statistical Analysis. A linear regression model was also used to examine the association between intramuscular fat content and expression level using the R Statistical Package.[9] This resulted in the equation

$$\text{expression}_{ij} = \mu + \text{fat}_i + \text{age}_{ij} + \text{e}_{ij}$$

where expression is an expression value of the each gene and protein, μ is the overall mean, fat is the intramuscular fat content (%) of each animal, and age is the covariate, slaughtering age (months). To determine major patterns and relationships in the gene expression data, we performed principal component analysis (PCA) for the genes.

RESULTS

Meat Quality Characteristics between Groups. Ten steers with low intramuscular fat content (low-marbled group) and 10 steers with high intramuscular fat content (high-marbled group) were used in a linear regression model to identify an association between the expression of mRNA and protein and intramuscular fat content. Of the traits associated with meat quality, lightness (L^*) value, carcass weight, intramuscular fat content, and Warner−Bratzler shear force were significantly different between the two groups. The average intramuscular fat content in m. longissimus tissue for the low-marbled group was 7.4 ± 1.5% and that for the high-marbled group was 23.5 ± 2.8% (Table 2). To confirm the differences between the low- and high-marbled groups, we implemented a linear mixed model to fit animal nested within age as a random effect. As shown in Table 2, lightness (L^*) value ($P < 0.01$), intramuscular fat content ($P < 0.001$), and Warner−Bratzler shear force ($P < 0.05$) were significantly different between groups. There were no significant differences between the low- and high-marbled groups for the properties of redness (a^*), yellowness (b^*), or carcass weight (Table 2).

Expression and Pathway Analysis. In this study, we investigated the gene expression of HSPB1 in m. longissimus muscles with divergent intramuscular fat contents. First, we examined the gene expression of CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) as indicators of fat deposition in muscle. The C/EBPα and PPARγ gene expression levels showed positive correlations with intramuscular fat content ($P < 0.05$ and 0.01, respectively) in the m. longissimus tissue of Korean cattle (Figure 2A). To determine whether HSPB1 was associated with intramuscular fat content, we investigated gene and protein expression between groups using real-time PCR and immunoblotting. As shown in Figure 1, expression level for both gene and protein was significantly expressed with decreasing intramuscular fat content ($P < 0.05$). Our results also show that the protein expression of HSPB1 was positively correlated with WB shear force ($R^2 = 0.547$, $P < 0.05$).

As shown in Table 3, to identify upstream proteins associated with the HSPB1 gene, we performed pathway analysis using Pathway Studio software. We detected 20 genes that act as upstream regulators of HSPB1. The gene expression of the two genes (FAS and AGT) showed significantly negative correlation with intramuscular fat content ($P < 0.05$) in the m. longissimus tissue of Korean cattle (Figure 2A). We also applied PCA to the gene expression data set. The relationships among these genes were illustrated by PCAs. As shown in Figure 2B, a biplot revealed that principal component 1 (PC1) and PC2 explained 58 and 18% of the total variance, allowing most of the information to be visualized in two dimensions. PC1 clearly separated samples into two groups, low- and high-marbled groups. In this analysis, the first PC illustrated the link among HSPB1, FAS, and AGT genes, which located on the positive regions of PC1. Moreover, these three genes (HSPB1, FAS, and AGT) were negatively related with the C/EBPα and PPARγ genes.

DISCUSSION

We investigated the gene expression of heat shock protein (HSP) with regard to intramuscular fat content in this study.

Table 3. Pathway Studio and Expression Analysis of Upstream Regulator Genes of the HSPB1 Gene

					expression[a]		
relation[b]		gene	full name	MedLine ref	low	high	*P* value[c]
expression	positive	FAS	TNF receptor superfamily, member 6	10978337, 11003656	1.3	0.9	**0.036**
		F2	coagulation factor II (thrombin)	16527988, 12181122, 10356108	2.4	2.3	0.351
		TGFB1	fransforming growth factor, β 1	11909638, 12706291, 12649267, 16055503, 18407296	0.6	0.7	0.889
		FGF2	fibroblast growth factor 2	11368160, 14699143	1.1	0.8	0.620
		EDN1	endothelin 1	10600794, 11701442, 11960785	0.6	0.8	0.296
		AGT	angiotensinogen	15087290, 11751700, 17895590, 11799090, 15270082, 128001926, 15001451	1.3	0.8	**0.011**
	negative	IFNG	interferon, γ	12700631, 18497994	1.1	1.2	0.530
	unknown	ESR1	estrogen receptor 1	8923463, 8095853, 15833836, 15123601, 10976925, 9328340	0.9	1.0	0.460
		STAT3	signal transducer and activator of transcription 3	16527988, 16322258, 17974989	0.7	0.6	0.098
		HSP90AA1	heat shock protein 90 kDa α (cytosolic), class A member 1	16492569, 17409432	0.5	0.7	0.825
promoter binding	positive	STAT1	signal transducer and activator of transcription 1, 91 kDa	12917439	1.2	1.0	0.628
		ATF5	activating transcription factor 5	17606386, 19046351	0.7	0.5	0.407
		PSIP1	PC4 and SFRS1 interacting protein 1	11687539, 15371438, 11677226	1.3	1.0	0.537
		ATF3	activating transcription factor 3	12832543, 17652582	0.7	0.5	0.098
		HSF1	heat shock transcription factor 1	12356732, 17623484, 12917439, 16492569, 131100329, 131101944, 15339936	1.2	1.5	0.135
		POU4F2	POU class 4 homeobox 2	15833836, 160108562	2.4	2.4	0.297
	unknown	NR1H4	nuclear receptor subfamily 1, group H, member 4	9223286	1.7	1.4	0.541
		SP1	Sp1 transcription factor	8923463, 9328340, 10976925	1.3	1.0	0.565
		POU4F1	POU class 4 homeobox 1	18368538, 15833836	1.7	1.1	0.161
		HSF2	heat shock transcription factor 2	17915561	0.6	0.6	0.462

[a] Expression shown as the mean of normalized expression value of each gene within low- and high-marbled group. [b] Expression and promotor binding indicate that the regulator changes the expression level and binds the promoter of the target. [c] *P* value was calculated using the regression analysis.

HSPs have essential roles in the synthesis, transport, and folding of proteins[10] and are often referred to as molecular chaperones.[11] In addition to HSPs, the crucial role of a variety of stress response proteins, especially those associated with oxidative stresses, have been emphasized in cell homeostasis and development. In adipogenesis, free radical formation has been demonstrated to inhibit fat cell formation; in particular, mitochondrial reactive oxygen species (ROS) are substantially involved in adipogenesis.[12,13] The increase in ROS content during stress is a signal mechanism of the synthesis of HSP families.[14] A recent study found evidence that HSPs also interact with adipogenic processes, thereby building complexes with glucocorticoid receptors and PPARs.[15−17] Furthermore, many studies have revealed that heat shock proteins, especially DNAJA1 and HSPB1, are correlated with beef tenderness and are negatively associated with WB shear force.[18,19] These papers suggest that down-regulation of HSP27 by encoded HSPB1 can actually accelerate actin disorganization or degradation, a process that is closely related to meat tenderness.

As many studies have suggested, adipocyte differentiation may be induced by both IGF-1 and insulin.[20−22] Insulin is known to act through the insulin-like growth factor 1 (IGF-1) receptor. Other papers have demonstrated that HSPB1 interacts with insulin-like growth factor receptor (IGFR)-1 and its signal transducer, the serine/threonine kinase protein, Akt, thereby influencing insulin sensitivity.[23,24] The IGFR-1 also triggers intracellular cascades that induce or repress transcription factors that modulate adipogenesis. Matsuzaki et al.[25] reported that plasma IGF-1 levels were lower in Japanese black cattle (Wagyu), which deposit much higher amounts of intramuscular fat, than in Holstein cattle. Previous in vitro studies have demonstrated the activation of mitogen-activated protein kinase (MAPK) by IGF-1 in hormone-responsive cells.[26] HSPB1 was also shown to be a terminal

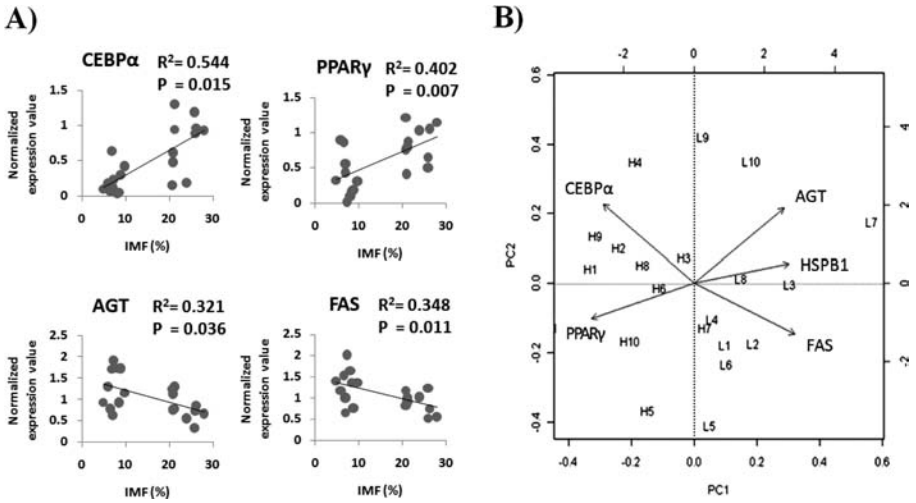

Figure 2. Gene expression profiles and relationships: (A) gene expression for CCAAT/enhancer-binding protein α (C/EBPα), peroxisome proliferator-activated receptor γ (PPARγ), TNF receptor superfamily, number 6 (FAS), and angiotensinogen (AGT) gene in muscle with intramuscular fat (IMF) content; (B) biplot of the first two principal components. L and H represent low- and high-marbled samples in the plot, respectively.

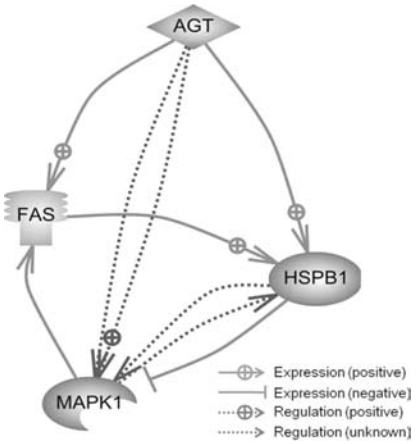

Figure 3. Pathway analysis of genes. Direct interaction analysis between the genes of our interest suggested that they form a part MAPK signaling pathway. The direct-interaction relationship indicates unknown, negative, and positive between the direct-interacted genes. Each arrow indicates interactions between genes.

substrate in the MAPK cascade; that is, HSPB1 was involved in the MAPK signaling pathway.[27] These findings suggest that HSPB1 may indirectly modulate adipogenesis through interactions with IGFR-1 and MAPKs signaling pathway. Interestingly, our results showed that HSPB1 gene and protein expression was significantly different between the low- and high-marbled groups and was significantly correlated with intramuscular fat content. Recently, HSPB1 was reported in human mesenchymal stem cells (MSCs);[28,29] especially, down-regulation of HSPB1 expression was reported in bovine MSCs[30] during the adipogenic differentiation. These agree with our data that HSPB1 may be involved in the modulation of adipocyte differentiation and metabolism.

TNF receptor superfamily number 6 (FAS), a 45 kDa protein that belongs to the tumor necrosis factor (TNF) receptor superfamily, is known as an inducer of apoptosis.[31] The tumor necrosis factor receptor superfamily (TNFRSF), more than 20 members of which have been identified in mammalian cells, plays an important role in the regulation of diverse biological activities.[32,33] Signaling via TNFRSF members that associate with TNF-receptor associated factor (TRAF) activates a number of intracellular signaling pathways, including those involving activation of NF-κB and MAP-kinases.[34] Recent findings in human MSCs revealed that TNFRSF19 expression was negatively regulated by adipogenic transcription factor CCAAT/enhancer-binding proteins (C/EBP).[35] Another paper demonstrated that several members of the C/EBP family were down-regulated by MAPKs in 3T3-L1 differentiation.[36] Our current study found that FAS was negatively correlated to intramuscular fat content. These findings suggest that the FAS gene may be one of the key genes controlling adipogenesis through a MAPK signaling pathway.

Angiotensinogen (AGT) is a precursor of angiotensin II and a major secretory product of the liver and adipocytes. Angiotensin II was proposed as a trophic factor in white adipose tissue growth and development. Previous papers have indicated that angiotensin II both inhibits[37,38] and promotes[39−41] adipocyte differentiation in different experimental models. These reports demonstrated that the effects of angiotensin II on adipocyte metabolism and differentiation are not conclusive. More recently, Fuentes et al.[42] investigated the antiadipogenic effect of angiotensin II on human preadipose cells and showed that the expression of the phosphorylated PPARγ gene was highly increased by treatment with angiotensin II through MAPK/ERK$_{1,2}$ activation. In the current study, the AGT gene was negatively correlated with intramuscular fat content. Therefore, these results collectively suggest that AGT might be involved in the phenotypic differences between low- and high-marbled cattle.

Interestingly, the results of our pathway analysis were consistent with the previous finding[27,33,42] that these genes might be directly or indirectly involved in the MAPK signaling pathway (Figure 3). In general, the process of adipocyte differentiation (adipogenesis) is initiated by the production of two key transcriptional factors, CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ). Also, adipogenesis is inhibited through the MAPK-mediated phosphorylation of PPARγ.[43,44] In the present study, expression levels of C/EBPα and PPARγ genes were positively associated with intramuscular fat content, but negatively related with HSPB1,

FAS, and AGT gene expressions. These findings suggest that the HSPB1, FAS, and AGT genes might negatively control adipogenesis via the MAPK signaling pathway.

In conclusion, we found that three genes (HSPB1, FAS, and AGT) were negatively related to intramuscular fat content in m. longissimus tissue of Korean cattle. These results suggest that HSPB1, FAS, and AGT may be good candidate genes associated with intramuscular fat content in the m. longissimus tissue of Korean cattle.

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Funding Sources

This study was supported by 2010 Post Doctoral Course Program and Agenda (20091FHT020710485 and 200901FHT020710399) of the National Institute of Animal Science, Rural Development Administration, Republic of Korea.

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