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Functional study of Villin 2 protein expressed in longissimus dorsi muscle of Korean native cattle in different growth stages

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The aim of this study was to investigate protein profiles related to the induction of adipogenesis within the bovine longissimus dorsi muscle (BLDM) by proteomic analysis. We analyzed BLDM proteins at different growth stages to clarify the physiological mechanisms of marbled muscle development in 20 head of Korean native cattle (11 month: 10 head, 17 month: 10 head). BLDM proteins were analyzed by two-dimensional electrophoresis and image analysis. Villin 2 was specifically identified by mass spectrometry and a protein search engine. Villin 2 protein expression in BLDM decreased during the fat development stage in test steers. In a Western blot cell culture study of spontaneously immortal bovine muscle fibroblasts, the abundance of Villin 2 was shown to be down-regulated during differentiation into muscle. In 3T3-L1 mouse embryonic fibroblasts, Villin 2 was decreased during differentiation into adipocytes. The results suggest that Villin 2 may be related to the induction of transdifferentiation and adipogenesis in bovine longissimus dorsi muscle. [BMB reports 2012; 45(2): 102-107]

INTRODUCTION

Meat tenderness varies with muscle properties such as fiber characteristics, glycogen content, collagen, and intramuscular fat. In general, the content and composition of intramuscular fat contributes to the determination of tenderness and flavor (1). In addition, the genotype of animals considerably influences their muscle properties. Despite some detailed knowledge of the structure of several structural proteins of bovine breeds, whether the developmental stage of several structural proteins in Korean native steers are directly responsible for tenderness and flavor is unknown.

Korean native cattle (Hanwoo) are known as a breed whose accumulation of intramuscular fat is genetically excellent. Generally, intramuscular fat increases as a steer grows; adipogenesis (formation of fat cells) occurs between 11-13-months-of-age in Korean native cattle and development of fat continues with growth. In this process, preadipocytes change to adipocytes (fat cells), causing adipocyte lipid metabolism and adipocyte lipid hypertrophy (2, 3).

While the mechanism of adipogenesis in skeletal muscle is not clear, recent data has linked adipogenesis with transdifferentiation of mesenchymal stem cells (MSCs) (4). MSCs are most prevalent in the early developmental stages of skeletal muscle and decrease according to the progress of growth (5).

In myogenesis, muscle cells and adipocytes transdifferentiate into MSCs. In this process the myogenin and MyoD myogenic markers are expressed (6-9). However, during the transdifferentiation into adipocytes, adipogenic transcription factors including CCAAT/enhance binding protein (C/EBP) α and peroxisome proliferator-activated receptor (PPAR) γ participate in the process at the preadipocyte stage (2, 4, 10).

Adipogenesis is controlled by genetic, nutritional, and environmental factors. Through key signaling pathways, the function of MSCs changes from myogenesis to adipogenesis, increasing intramuscular fat (1). Meanwhile, Wingless and Int (Wnt)/β-catenin signaling controls the differentiation of MSCs in the embryo; myogenesis increases as Wnt/β-catenin increases and adipogenesis decreases (5). Also, it was reported in Du et al. (2010) that control of the stage of adipogenesis is related to factors that include Hedgehog signaling, bone morphogenetic proteins (BMP) signaling, and AMP-activated protein kinase (AMPK) (11).

However, the physiological mechanism related to the development of marbled muscle is unclear (1). Although there is almost nothing known about the stimulus for marbling, once marbling begins, the muscle may undergo characteristic changes that include the expression of specific proteins. The present

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study identified the proteins of bovine longissimus dorsi muscle (BLDM) expressed at different stages of growth to clarify the physiological mechanism underlying development of marbled muscle in Korean native cattle.

RESULTS AND DISCUSSION

Protein expression during fat and muscle development in castrated Korean native steers

Generally, organ growth in castrated Korean native steers begins at 2.7-months-of-age, reaches a maximum growth at 11-months -of-age, while fat growth begins at 12.4-months-of-age and attains its most vigorous growth at 17-months-of-age. The average monthly growth of muscle and fat are 3.84 kg and 3.85 kg, respectively. Intramuscular fat growth is especially pronounced before and after 17-months-of-age. Presently, the weight of 17-month-old cattle (425.6 ± 22.7 kg) increased 1.48-fold more than the weight of 11-month-old cattle (287.2 ± 33.0 kg), and the area of the BLDM increased 1.4-fold. Ultrasound examinations confirmed that back fat thickness and marbling score increased 2-fold in 17-month-old cattle (0.6 ± 0.25 cm^2), as much as in 11-month-old cattle (0.3 ± 0.17 cm^2).

In general, there are several theories for the accumulation of intramuscular fat. The current favored theory posits the differentiation of stem cells into muscle that transdifferentiates into fat. Accordingly, the present study assessed protein changes that occur in the stages from muscle accumulation to fat accumulation by investigating the expression of protein in the BLDM tissue of 11-month-old cattle, when muscle development is the most vigorous, and 17-month-old cattle, in which a rapid increase of intramuscular fat is occurring. Our goal was to acquire basic data regarding muscle development and fat accumulation mechanisms.

To identify protein(s) uniquely expressed at the time of muscle and fat development, 2-DE analysis was conducted on pooled BLDM tissue. Proteins whose contents were ≥5-fold different were identified. Among the identified proteins, one was expressed at the time of muscle development and decreased at the time of fat development (Fig. 1); MALDI-TOF MS identified the protein as Villin 2 (Table 1).

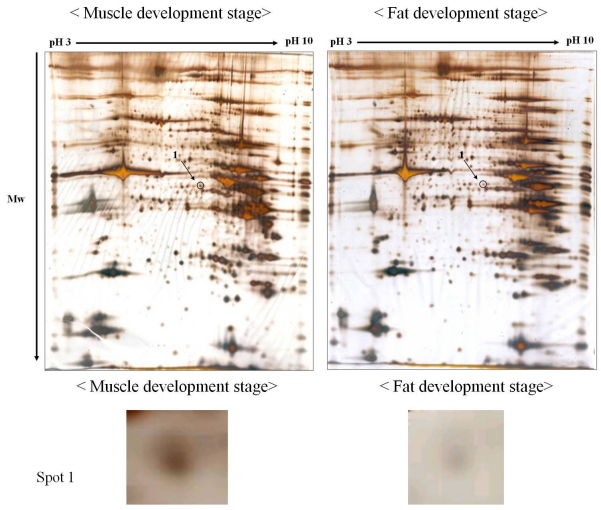

Fig. 1. Appearance of a representative silver-stained 2-DE gel of BLDM tissue obtained at the time of muscle development and fat development.

Expression of Villin 2 protein during Hanwoo growth and change in marble score

2-DE revealed that Villin 2 expression tended to increase at the time of Hanwoo muscle development (11-months-old) and decrease at the time of fat development (17-month-old). To verify this result, the expression of Villin 2 in BLDM tissue at each growth stage was investigated. Castrated Hanwoo (n = 10) were randomly assigned, and BLDM tissues were collected from 6-, 11-, 17-, and 24-month-old cattle. The protein was extracted and pooled. Villin 2 protein was analyzed by Western blot. The amount of expressed Villin 2 significantly decreased according to growth (Fig. 2a) ($P < 0.05$). Especially, expression of Villin 2 protein was the highest in 6-month-old cattle and continuously decreased after 17-months-of-age ($P < 0.05$) (Fig. 2a). The result verified that the expression of Villin 2 protein decreased according to the increased accumulation of fat in muscle tissue. Also, we examined the change in expression of Villin 2 protein based on the difference in longissimus dorsi marbling levels. After comparing the expression aspects between candidate proteins from a high marble score group (=4.7) and low marble score group (=2), the results did not show any statistically significant difference, although a decreased amount of Villin 2 protein was evident in the high marble score group (Fig. 2b). The collective results support the view that Villin 2 protein is expressed in the early growth phase of Hanwoo, at the time of muscle development, and decreases according to the progress of muscle development, and especially according to the increase of intramuscular fat accumulation.

Table 1. Characterization of differentially expressed proteins between the different age groups (11- and 17-month-old) using MALDI-TOF MS

Spot no	Protein name	Accession no	Sequence coverage (%)	Extimated Mw (Da/pI)	MOWSE score	Ratio[a]
1	Villin 2	27806351	21	68761/6.1	1.465e + 04	3.7

[a]Ratio value of the 11- to 17-month-old group.

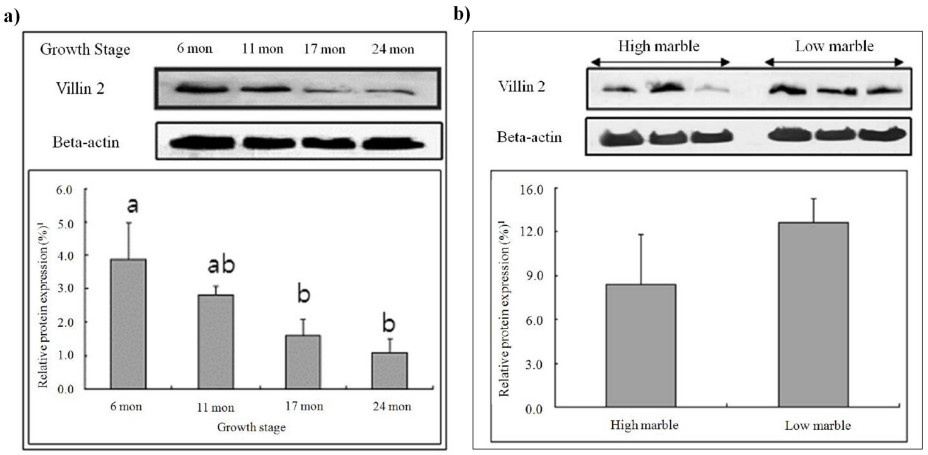

Fig. 2. Change of Villin 2 protein expression. (a) Changes in expression of Villin 2 protein in BLDM tissues during different growth phases of Korean native steers. (b) Changes in expression of Villin 2 protein between high marble score group and low marble score group in castrated Korean native steers. All data represent the mean ± SE of 3 determinations. [1]Villin 2/beta-actin ×100. ab, $P < 0.05$.

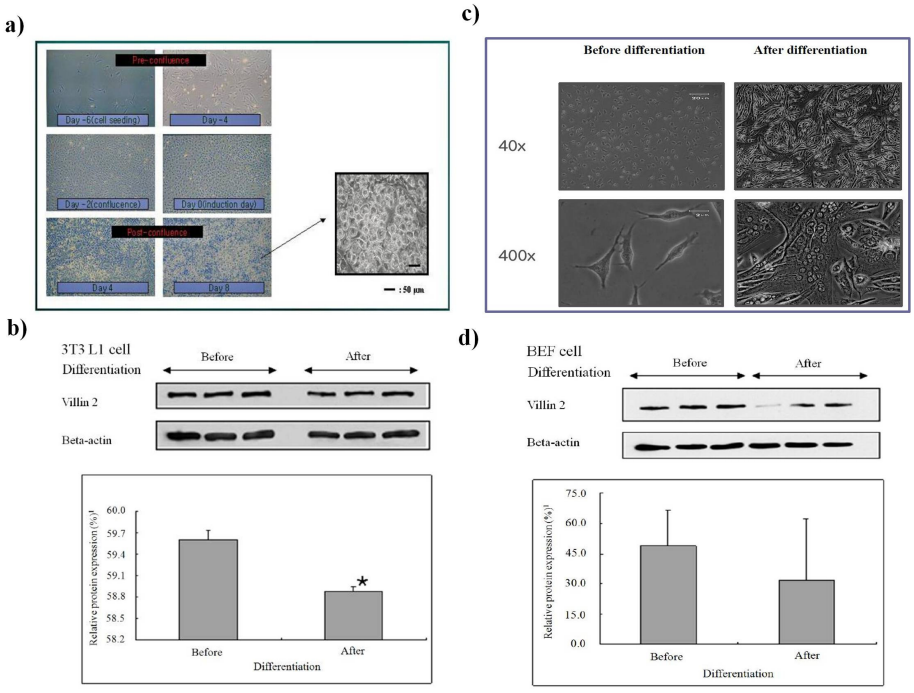

Fig. 3. Changes in expression of Villin 2 protein according to the progress of adipogenesis in preadipocytes of 3T3L-1 cells and myogenesis in myoblasts of BEF cells. (a) Images before and after differentiation of 3T3L-1 cells. (b) Western blot result of Villin 2 protein before and after differentiation of 3T3L-1 cells. (c) The image before and after differentiation of BEF cells. (d) Western blot result of Villin 2 before and after differentiation of BEF cells. *$P < 0.05$.

Functional analysis of Villin 2 protein

To verify the growth-related characteristic of Villin 2 protein, the quantity of Villin 2 before and after differentiation of muscle cells and adipocytes was examined using 2 types of fibroblasts (BEFS and 3T3-L1). Images acquired before and following the induced differentiation of 3T3-L1 preadipocytes for 8 days in differentiation maintenance medium are shown in Fig. 3a. Preadipocyte differentiation into adipocytes was apparent. In addition, when the amounts of expression of Villin 2 protein before and after differentiation were compared, a significant decrease in Villin 2 expression after differentiation was evident ($P < 0.05$) (Fig. 3b). Also, the results obtained before and after BEF differentiation confirmed that fibroblasts that grew as fibrocytes before differentiation, grew as muscle cells after differentiation (Fig. 3c). Comparison of the expression of Villin 2 protein before and after differentiation revealed decreased protein after differentiation in the mature muscle cells (Fig. 3d). This result is similar to the result of a previous 2-DE study that reported that phosphorylated Villin 2 protein was expressed during myoblast growth of C2C12 cells and was not expressed during early myogenesis (12).

Generally, Villin 2 protein is called ezrin, and it is one the proteins that belong to ezrin-radixin-moesin (ERM) cytoskeleton-associated protein family (13-15). Villin 2 provides a crucial morphological link between the cell plasma membrane and the

actin-based cytoskeleton (16, 17), maintaining cell shape and polarity, and participating in cell migration, signaling, growth regulation, and differentiation (18, 19). Also, Villin 2 interacts with membrane proteins such as CD44, CD43, intercellular adhesion molecule-1 and -2, and phosphatidylinositol [4,5]-biosphosphate, controlling cell adhesion (18, 20-24). Villin 2 also interacts with the cell adhesion molecules E-cadherin and β-catenin, and controls cell-cell and cell-matrix adhesion (25). In addition, Villin 2 participates in controlling the growth of cancer cells and, as an important metastasis-associated molecule, it is over-expressed in various tumors (26-30). Microarray array analysis has revealed increased levels of Villin 2 expressed in conjunction with up-regulation of the gene for leptin, which plays a role in mammary tumor growth (31). Also, in colon cancer cells (CRCs), Villin 2 forms a complex between L1CAM and IKB, and participates in L1CAM-mediated metastasis with nuclear factor-kB signaling (32). L1CAM exists in CRCs as a target for β-catenin-T-cell-factor (TCF) signaling (33, 34).

β-catenin inhibits adipogenesis in cells through wnt signaling and increases myogenesis (35). Likewise, testosterone inhibits adipogenesis through the androgen receptor and β-catenin interaction (36). On the one hand, androgen up-regulates the expression of Villin 2 through the androgen receptor (37). In this aspect, Villin 2 and androgen receptor have an intimate relationship. This suggests the possibility that Villin 2 acts through the androgen receptor by interacting with β-catenin to control cell adhesion and inhibit adipogenesis.

Also, the expression of Villin 2 in the preadipocyte stage of adipogenesis can be influenced by testosterone. Presently, the decrease in Villin 2 expression in 3T3-L1 cells before and after differentiation may have reflected the lack of influence of added testosterone. In addition, Villin 2 was reported as a down- regulated protein in MSCs (38).

Therefore, in castrated steers, because the expression of testosterone is inhibited, the inhibition of myogenesis and increase of adipogenesis along with growth could be strongly related to Villin 2 expression. In addition, due to the decrease of testosterone, the anti-adipogensis function of β-catenin linked with Villin 2 could be inhibited, which could facilitate the transdifferentiation from muscle tissue to adipocytes in Hanwoo. However, more specific research is needed on the role of Villin 2 related to the control of wnt signaling and adipogenesis. These studies will be important for interpreting the association of Villin 2 with transdifferentiation of muscle tissue in Koran native steers.

MATERIALS AND METHODS

Animals and sample preparation

Korean native steers (n = 20) at 2 physiological stages, 11-months-of-age (n = 10) and 17-months-of-age (n = 10) were studied. Skeletal muscle tissue was obtained from the bovine longissimus dorsi muscle (BLDM) of each animal at a depth of near rib 13 by biopsy after local anaesthesia. Each sample was used for proteome analysis. In another experiment, skeletal muscle tissue was similarly acquired from 10 Korean native steers at 4 growth stages (6-, 11-, 17-, and 24-months-of-age) for use in Western blot examinations of protein expression. In a third experiment, six 24-month-old steers were divided into 2 groups: 3 bovine individuals with higher marbling scores ($\geq$6) and 3 with lower marbling scores ($\leq$3), according to a Korean marbling grade system in which marbling grade consists of 9 evaluation values. Samples of BLDM were obtained at the junction between the 12th and the 13th lumbar vertebrae within 30 min after slaughter, as described above. The obtained tissues were immediately placed in liquid nitrogen and stored at −80°C for subsequent proteomic analysis.

Frozen muscle tissues (300 mg) were homogenized in lysis buffer (3 mL) containing 7 M urea (GE Healthcare, Buckinghamshire, UK), 2.8 M thiourea (Sigma-Aldrich, St. Louis, MO, USA), 4% (w/v) 3-3 (cholamidopropyl) dimethylammonio-1- propanesulfonate (CHAPS; GE Healthcare), 65 mM dithiothreitol (DTT; GE Healthcare), 2% immobilized pH gradient (IPG) buffer (pH 3-10 NL; GE Healthcare), 1 × Nuclease Mix (GE Healthcare), 1 × protease inhibitor (GE Healthcare), and 0.002% (w/v) bromophenol blue (Sigma-Aldrich). The homogenate was stirred every 5 min for 1 h, after which the samples were centrifuged at 14,000 × g for 20 min at 20°C. Aliquots of the supernatant were then stored at −80°C until analysis. The protein content was determined using a PlusOne 2-D Quant Kit (GE Healthcare) with bovine serum albumin (BSA) as the standard.

Two-dimensional gel electrophoresis and image analysis

Two-dimensional electrophoresis (2-DE) was conducted as previously described (39). The solubilized protein samples (100 μg) were applied onto 18 cm Immobiline DryStrips (pH 3-10 NL; GE Healthcare). After completion of 2-D SDS-PAGE, each gel was stained using a PlusOne Silver Staining Kit (GE Healthcare), according to the manufacturer's instructions. The silver stained gels were scanned using a Power Look 2100XL scanner equipped with Magic Scan version V5.2 software (UMAX Technologies, Inc., Dallas, Texas, USA). Spot detection, pair matching, and normalization were performed using ProteomWeaver software (Definiens, Munich, Germany). The 2-DE analyses of 11-month-old cattle (n = 10) and 17- month-old cattle (n = 10) were performed using 20 gels. For comparative analysis, the relative densities of individual spots were analyzed and compared between the 2 groups. The spots that were present in the total gels were considered as the candidate spots. The differentially expressed spots with at least a 2-fold change in intensity were used for the comparison.

Protein identification

Protein spots were subjected to in-gel trypsin digestion. The mass spectra of peptides generated by in-gel digestion were analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). To identify the proteins, the peptide masses from MALDI-TOF MS were matched with the theoretical peptides of proteins in the SwissProt and NCBI databases using MASCOT software. MS patterns of tryptic peptides derived from each spot were searched against protein

sequences present in the NCBInr and EST databases using the MASCOT (http://www.matrixscience.com) search program.

Cell culture

Spontaneously immortalized bovine embryonic fibroblasts (BEFS) (40) and 3T3-L1 mouse embryonic fibroblasts (adipose like cell line) were grown and maintained in growth medium (GM) consisting of high-glucose Dulbecco's modified Eagle medium (HyClone, Logan, UT, USA), 10% (w/v) fetal bovine serum (HyClone), 1% (w/v) penicillin-streptomycin (Gibco, Franklin Lakes, NJ, USA), and 1% (w/v) L-glutamine (Gibco). To induce myogenic differentiation, confluent BEFS cells were exposed to a differentiation medium consisting of GM supplemented with 2% (w/v) horse serum (HyClone), 10 μg/ml insulin, and 1 μg/ml doxycycline (Calbiochem, San Diego, CA, USA). The cells were maintained in the differentiation medium for 4 days to induce complete differentiation. Mouse 3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were maintained in GM. Differentiation was induced in 100% confluent 3T3-L1 cells by incubation in differentiation-induction medium (GM containing 1 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine) for 2 days. Next, cells were maintained in differentiation maintenance medium (GM supplemented with 1 μg/ml insulin and 10% FBS) for 8-14 days. The differentiation maintenance medium was changed every 2 days until the cells were harvested.

The fibroblast or preadipocyte and differentiated cellular lysates in lysis buffer (20 mm Tris pH 7.5-8.0, 150 mM sodium chloride, 1 mM EDTA, and 1% Triton X-100 containing protease inhibitors) were subjected to Western blot analyses.

Western blotting

Protein samples (30 ug) were subjected to 10% (w/v) SDS-PAGE and the resolved proteins were transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.01% Tween 20) containing 5% skim milk and then incubated for 3 h at 4°C with one of the following primary antibodies: mouse monoclonal antibody to a synthetic peptide corresponding to amino acids 1-15 of Xenopus laevis β-actin (ab6276, 1 : 5,000, 42 kDa; Abcam, Cambridge, UK) or mouse monoclonal antibody [3C12] to human Villin 2 (ab4069, 1 : 300, 81 kDa; Abcam). The membranes were then washed 3 times with 1 × TBST, after which they were incubated with the appropriate secondary horseradish peroxidase (HRP)-conjugated rabbit polyclonal antibody to mouse IgG (ab6728, 1 : 2,000; Abcam) for 3 hr at 4°C .The proteins on the membrane were visualized using an enhanced chemiluminescence system plus detection kit (GE Healthcare), after which they were exposed to X-ray film (Fujifilm Corporation, Tokyo, Japan) for 1-3 min. The films were then scanned and the bands were quantified using ProteomWeaver software (DEFINIENS, Munich, Germany); the protein levels were normalized by comparison to β-actin signals on the same membrane.

Statistical analyses

The Student's t-test was used for analysis of data from in vitro experiments conducted before and after cell differentiation, and for data comparing the high marble and low marble groups. Analysis of variance was conducted using SAS version 8.1 (SAS Institute, Cary, NC, USA) for data on the expression of Villin 2 protein during growth. The statistical significance between the processes was analyzed by Duncan's multiple probation of GLM (general linear model) procedure.

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