Enhancement of adipogenesis and fibrogenesis in skeletal muscle of Wagyu compared with Angus cattle

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ABSTRACT: Intramuscular fat and collagen content are major factors affecting beef quality, but mechanisms regulating intramuscular adipose and connective tissue deposition are far from clear. Japanese Wagyu cattle are well known for their extremely high marbling. The objective of this study was to evaluate intramuscular fat (IMF) and collagen deposition in the muscle of Wagyu compared with Angus cattle. Animals were managed under the same condition and slaughtered at an averaging $585 \pm$ 12.1 kg of BW. Samples of sternomandibularis muscle were collected from Wagyu (n = 3) and Angus (n = 3) for molecular and histological investigations of adipogenesis and fibrogenesis. With exception of C/EBP β (P = 0.2864), the expression of the adipogenic markers C/EBP α (P = 0.008), PPARy (P = 0.028), and zip finger protein 423 (Zfp423; P = 0.047) in Wagyu were greater than in Angus

muscle, which was consistent with greater IMF deposition in Wagyu (P < 0.05). In addition, more adipocytes and preadipocytes were detected intramuscularly in Wagyu cattle. Similarly, fibrogenesis was also enhanced in Wagyu, with a greater expression of fibroblast growth factor (FGF)-2 (P = 0.028), FGF receptor 1 (P = 0.030), transforming growth factor (TGF)- β (P = 0.028), collagen I (P = 0.012), and collagen III (P = 0.025). Similarly, Wagyu muscle had greater collagen content (P = 0.002) and decreased collagen solubility (P = 0.005). In addition, muscle fiber diameter was larger (P < 0.0001) in Wagyu than in Angus cattle. These results clearly show that both IMF and collagen contents are enhanced in Wagyu cattle and more adipogenic cells are detected in Wagyu muscle, indicating intramuscular adipogenesis is enhanced in Wagyu compared with Angus muscle.

Key words: adipogenesis, Angus, beef, collagen, fibrogenesis, intramuscular fat, Wagyu, zip finger protein 423

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INTRODUCTION

According to recent surveys of beef producers by the National Cattlemen's Beef Association, marbling and tenderness were identified as the top beef quality problems (McKenna et al., 2002; Garcia et al., 2008). Marbling becomes a major quality problem because of the selection for high lean growth, which results in overall reduction of fat accumulation, including intramuscular fat (**IMF**; Du et al., 2010; Albrecht et al., 2011), which is critical for the palatability of meat (Savell et al., 1987; Du et al., 2010). The most optimal solution to this problem is to enhance intramuscular adipose deposition

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without increasing fat deposition in other depots, which necessitates the understanding of molecular and cellular mechanisms regulating IMF development. The presence of connective tissue, primarily in the form of collagen fibrils and the degree of intermolecular cross-linking, contributes to the background toughness of meat (Duarte et al., 2011). Both marbling adipocytes and connective tissue fibrils such as collagen are located in the extracellular matrix juxtaposed to skeletal muscle

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Although muscle cells, adipocytes, and fibroblasts are originated from the same pool of mesenchymal progenitor cells, myogenic progenitor cells and fibrogenic/adipogenic progenitor cells diverge early during development (Du et al., 2013); fibroblasts and adipocytes share immediate progenitor cells, socalled fibro/adipogenic cells, located in the evolving

fibers.

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extracellular matrix of primordial muscle fibers (Joe et al., 2010; Uezumi et al., 2010; Uezumi et al., 2011; Du et al., 2013). Enhancement of adipogenic differentiation of these progenitor cells increases the number of intramuscular adipocytes whereas fibrogenic differentiation promotes the synthesis of connective tissue in muscle.

Wagyu cattle produce extremely high marbling; in addition to its production value, it is a good animal model to study adipogenesis and lipid metabolism (Sturdivant et al., 1992; Lunt et al., 1993; Radunz et al., 2009). Indeed, the mRNA expression of adipocyte markers, such as PPAR γ , is greater in Wagyu compared with other cattle breeds (May et al., 1994; Yamada et al., 2007, 2009). However, intramuscular adipogenic differentiation, fibrogenesis, and the structure of adipose tissue of Wagyu cattle have not been studied. Wagyu and Angus cattle have been used for comparison by a number of previous studies (Cameron et al., 1993, 1994; Lunt et al., 1993; May et al., 1993, 1994; Xie et al., 1996; Wertz et al., 2002; Chung et al., 2007; Lawrence et al., 2007; Rhoades et al., 2007; Radunz et al., 2009). As such, we investigated the difference in intramuscular adipogenesis and fibrogenesis between Wagyu and Angus cattle.

MATERIAL AND METHODS

Animal use was approved by the Animal Care and Use Committee of Washington State University (Protocol: 04251).

Animals and Muscle Tissue Sampling

Wagyu (n = 3) and Angus (n = 3) steers with similar age (24 mo) and average BW of 585.0 ± 12.1 kg were obtained from the Beef Center of Washington State University (**WSU**) and harvested in WSU Meat Laboratory. Here, we only use 3 steers per breed because of the large marbling difference between Wagyu and Angus, which provides sufficient power to discern breed effects. Steers were managed under the same conditions. Animals were slaughtered by desensitization with a nonpenetrating stunner followed by exsanguination in compliance with USDA regulations. Immediately after exsanguination, samples of sternomandibularis muscle (50 g approximately) were collected from each animal, minced, and snap frozen in liquid nitrogen promptly.

Frozen samples were then powdered in liquid nitrogen, placed in cryovials, and stored at -80°C for real-time quantitative PCR and chemical analysis. Another small portion of samples was fixed in 4% fresh paraformaldehyde immediately after slaughter and then processed for paraffin embedding (Huang et al., 2010). We used sternomandibularis muscle because 1) it is a long, uniform muscle clearly separated from the surrounding connective tissue, reducing sampling variation, 2) it only contains 1 major muscle bundle, without the presence of intermuscular connective tissue and fat, facilitating the separation of pure intramuscular adipose tissue, 3) it is highly accessible in harvested animals, and 4) the biological mechanisms regulating adipogenic differentiation are conserved across mammals, not to mention in the different muscles of the same animal; therefore, data obtained from sternomandibularis muscle are applicable to the LM, which is economically important.

Real-Time Quantitative PCR Analysis

Total RNA (1 μ g) was extracted from 0.5 g of powdered tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA), treated with deoxyribonuclease, and reverse transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The primer sets used are shown in Table 1. Real time-PCR was performed on a CFX ConnectTM Real-Time PCR detection system (Bio-Rad) using SYBR Green RT-PCR kit from Bio-Rad and the following cycle parameters: 95°C for 3 min and 40 cycles at 95°C for 10 s and 60°C for 30 s. The amplification efficiency was 0.90 to 0.99. After amplification, a melting curve (0.01 °C/s) was used to confirm product purity, and the PCR products were electrophoresed to confirm the targeted sizes. Results are expressed relative to 18S using the $\Delta\Delta$ Ctmethod (Bustin, 2002; Nolan et al., 2006).

Immunohistochemistry

Fixed tissues were sectioned (5 μ m) and deparaffinized in xylene followed by graded rehydration in ethanol (100, 95, 80, and 70%) and distilled water. Antigens were unmasked by hightemperature antigen retrieval [10 min boiling in 10 mM sodium citrate buffer (pH 6.0)] and allowed to cool slowly at room temperature. Sections were then washed twice (5 min each) with TBS/T solution (0.1%)Tween-20, 50 mM Tris-HCl, pH 7.6, and 150 mM NaCl) and incubated in blocking solution consisted of TBS/T and 10% BSA for 1 h at room temperature. Sections were incubated overnight at 4°C with antifatty acid binding protein 4 (FABP4) antibody (1:100 dilution) in TBS solution plus 1% BSA. Then sections were washed twice with TBS/T and then incubated with the Green fluorescent protein secondary antibody (1:1,000 dilution) in TBS with 1% BSA for 1 h at room temperature. Both primary and secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Sections were then washed 3 times with TBS for 5 min each, drained, and mounted in DAPI (4',6-diamidino-2-phenylindole) mounting medium

Tal	ble	1.	Primer	sets	used	in	this	stud	y
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	Gene	UniGene access	Forward	Reverse
Gene	abbreviation	code	sequence	sequence
Collagen type I, alpha 1	COL1A1	NM_001034039.1	CCACCCCAGCCGCAAAGAGT	ACGCAGGTGACTGGTGGGATGTC
Collagen type III, alpha 1	COL3A1	NM_001076831.1	GGCCCCCTGGAAAGGACGGA	CCCCGCCAGCACCACAACAT
Fibronectin 1	FN1	NM_001163778.1	GCGTGTCACCTGGGCTCCAC	CGGTGCCGGGCAGGAGATTT
Transforming growth factor, beta 1	TGFβ-1	NM_001166068.1	AGCCAGGGGGGATGTGCCA	TAGCACGCGGGTGACCTCCT
CCAAT enhancer binding protein, alpha	C/EBPa	NM_176784.2	TGCGCAAGAGCCGGGACAAG	ACCAGGGAGCTCTCGGGCAG
CCAAT enhancer binding protein, beta	C/EBPβ	NM_176788.1	CGGGCAGCACCACGACTTCC	CCCCAGTCGGCCCAGACTCA
Peroxissome proliferator actiated-receptor gamma	PPARγ	NM_001098905.1	TGGAGACCGCCCAGGTTTGC	AGCTGGGAGGACTCGGGGTG
Fibroblast growth factor 2 (basic)	FGF2	NM_174056.3	GGAGCATCACCACGCTGCCA	GTGGGTCGCTCTTCTCGCGG
Fibroblast growth factor receptor 1	FGFR1	NM_001110207.1	AGGAGGATCGAGCCCACGGC	CTTGCTCCGGCAAGGTCGGGG
Zinc finger protein 423	Zfp423	NM_001101893.1	GGATTCCTCCGTGACAGCA	TCGTCCTCATTCCTCTCCTCT
Myogenic differentiation 1	MYOD	NM_001040478.2	TTCCGACGGCATGATGGACTAC	TAAGTGCGGTCGTAGCAGTTCC
Myogenin (Myogenic factor 4)	MYOG	NM_001111325.1	TACAGACGCCCACAATCTGCAC	AGCGACATCCTCCACTGTGATG
18 S ribosomal	18S	NM_001033614	CCTGCGGCTTAATTTGACTC	AACTAAGAACGGCCATGCAC

for immunofluorescent observation using an EVOS fl fluorescence microscope (AMG, Bothell, WA).

Histochemical and Image Analysis

Muscle tissue sections (5 µm) were deparaffinized, rehydrated, and used for Masson's trichrome staining (Foidart et al., 1981), which stains muscle fibers red, nuclei black, and collagen blue (Huang et al., 2012). Muscle fiber sizes were measured using the ImageJ software (National Institute of Health, Baltimore, MD) and at least 200 muscle fibers per animal were measured (4 images per section and 5 sections per cattle). Similarly, to calculate FABP4:nuclei ratio and the intercellular space in the muscle sections, 4 images per section and 5 sections per cattle, were used for analysis using ImageJ software. To measure the intercellular area, fluorescent images were initially converted into 8-bit grayscale images and then submitted to a thresholding adjustment for a better identification of intercellular area. Then intercellular space was identified as a region of interest by using the tracing tool of the Image J software and the values were recorded. All images were analyzed in 200x magnification.

Collagen Content and Solubility Analysis

Powdered frozen muscle samples (0.1 g) were placed in 2 mL tubes and 400 μ L of distilled water was added in each tube. Tubes were capped and placed in a water bath at 80°C for 120 min. Tubes were then cooled at 4°C for 15 min and centrifuged for 20 min at 20,000 × g at 2°C using a 5424 R centrifuge (Eppendorf AG, Hamburg, Germany) and a FA-45-24-11 rotor (Eppendorf AG, Hamburg, Germany). After centrifugation, the supernatant and pellet of each sample were transferred to 2 different test tubes with screw caps and 2 mL of hydrochloric acid (HCl 6 *N*) were added to each tube. The tubes were capped and samples were hydrolyzed at 105°C for 16 h. After HCl digestion, samples were filtered through filter paper (Whatman #1) and an aliquot was removed and neutralized to pH 6.0 with NaOH. Aliquots were then used for hydroxyproline quantification as described by Woessner (1961). To determine collagen content, hydroxyproline amount was multiplied by 7.52 for the supernatant (soluble collagen) and 7.25 for the residual (insoluble collagen; Cross et al., 1973). Total collagen (mg collagen/g meat) was defined as soluble collagen plus insoluble collagen and percent solubility was calculated as soluble collagen divided by total collagen multiplied by 100.

Intramuscular Fat Content Analysis

The IMF content was determined using lyophilized muscle samples, the 50-g samples obtained at the harvest of the animal. Intramuscular fat content was quantified by using ether extract according to the method 920 of the Association of Official Analytical Chemists (AOAC, 1990).

Statistical Analysis

Statistical analysis was performed using SAS (SAS Inst. Inc., Cary, NC). For the gene expression and IMF content, a unilateral *t* test was used with the alternative hypothesis of Wagyu animals showing greater means than Angus animals for these dependent variables. For muscle fiber diameter, and collagen content and solubility, a bilateral *t* test was used. Statistical significances were considered at P < 0.05.



Figure 1. The mRNA expression of C/EBP α (A), C/EBP β (B), PPAR γ (C), and zip finger protein 423 (Zfp423) (D), and the intramuscular fat content (E) of Angus (\blacksquare) and Wagyu (\square) sternomandibularis muscle (*P < 0.05; n = 3; mean \pm SE).

RESULTS

Expression of Adipogenic Markers and Intramuscular Fat Content in Wagyu and Angus Muscle

Greater expression of late stage adipogenic markers, C/EBP α (P = 0.008) and PPAR γ (P = 0.028), was observed in Wagyu compared with Angus muscle (Fig. 1A and 1C). Similarly, the expression of early adipogenic marker, zip finger protein 423 (**Zfp423**), was also greater in Wagyu muscle (P = 0.047; Fig. 1D). On the other hand, no difference was observed for the expression of C/EBP β (P = 0.2864; Fig. 1B). In agreement with mRNA expression, the IMF content was greater in Wagyu muscle (P = 0.007; Fig. 1F).

Expression of Fibrogenesis Markers and Collagen Content and Solubility in Wagyu and Angus Muscle

The mRNA expression of fibrogenic related growth factors, including fibroblast growth factor (**FGF**)-2 (P = 0.028), FGF receptor (P = 0.030), and transforming growth factor (**TGF**)- β (P = 0.028), was greater in Wagyu compared with Angus muscle (Fig. 2A, 2B, and

2C). Consistently, the mRNA expression of fibrogenic proteins, including fibronectin (P = 0.002), collagen I (P = 0.012), and collagen III (P = 0.025), was also greater in Wagyu muscle (Fig. 3A and 3B). Total collagen content (P = 0.002) was greater in Wagyu muscle whereas the collagen solubility was greater (P = 0.0046) in Angus muscle (Fig. 3C and 3D).

Distribution of Collagen Fibers and Structure of Wagyu and Angus Muscle

The greater collagen content in Wagyu muscle was unexpected, which prompted us to examine the location of connective tissue inside muscle. As shown by trichrome staining, Wagyu muscle fibers were loosely packed with significant amount of spaces and collagen between muscle fibers and bundles whereas in Angus muscle, collagen was only detected in perimysiun and muscle fibers were densely packed without much space between muscle fibers (Fig. 3D and 3E).

Besides overall difference in collagen fiber distribution and muscle fiber packing density, we also noticed the presence of small and large muscle fibers in Wagyu muscle and the overall size of Wagyu muscle fibers appeared larger (Fig. 3). Indeed, the average



Figure 2. The mRNA expression of fibroblast growth factor (FGF)-2 (A), FGF receptor (FGFR) (B), fibronectin (C), and transforming growth factor (TGF)- β (D) in sternomandibularis muscle of Angus (\blacksquare) and Wagyu (\Box ; *P < 0.05; n = 3; mean \pm SE).

muscle fiber sizes were greater in Wagyu compared with Angus muscle (P < 0.0001) and the distribution of muscle fiber sizes was also wider in Wagyu muscle (Fig. 4A and 4B). The intramuscular space in Wagyu was also larger than that in Angus cattle (Fig. 4C). To further explore whether there was difference in myogenesis between Angus and Wagyu cattle, we analyzed the mRNA expression of myogenic differentiation factor D (**MyoD**) and myogenin (**MyoG**), and no difference was observed (Fig. 4C and 4D).

Presence of FABP4 Positive Cells in Wagyu and Angus Muscle

The adipogenic marker, FABP4, is expressed in both preadipocytes and adipocytes. To analyze the location of preadipocytes and adipocytes and their relative abundance, we conducted immunohistochemical analysis with FABP4 antibody. Using positive for both FABP4 and DAPI as criteria, the number of preadipocytes and adipocytes in Wagyu was greater than that of Angus cattle (Fig. 5). The number of these cells was likely vastly underestimated, especially for Wagyu cattle, because the section thickness was only 5 microns, which would miss many nuclei resulting in exclusion of many adipocytes and preadipocytes.



Figure 3. The mRNA expression of Angus (\blacksquare) and Wagyu (\square) sternomandibularis muscle. Collagen I and III (A), collagen content (B), collagen solubility (C), and representative images of muscle stained with Masson's trichrome (D and E) (*P < 0.05; n = 3; mean \pm SE). See online version for figure in color.



Figure 4. Frequency and size distribution of muscle fibers from Wagyu (dash line) and Angus (solid line) (A), average muscle fiber diameter (B) and intercellular space (C), and the mRNA expression of MyoD and MyoG (D and E) (*P < 0.05; n = 3; mean \pm SE).

DISCUSSION

Wagyu Cattle had Larger Muscle Fibers Compared with Angus Cattle

Wagyu cattle are well known for their extremely high marbling, but the mechanisms leading to the greater marbling is unclear. We hypothesized that this greater marbling is due to the changes in the differentiation of multipotent progenitor cells during skeletal muscle development. Muscle cells, adipocytes, and fibroblasts are derived from the same pool of mesenchymal progenitor cells, which are abundant in the skeletal muscle at early developmental stages but still present at significant levels in mature muscle (Du et al., 2010). Although the majority of these cells undergo myogenic differentiation, a significant portion differentiate into common progenitor cells committing to both adipocytes and fibroblasts, socalled fibro/adipogenic progenitor cells (Du et al., 2013).

Several studies have shown less muscularity in Wagyu cattle when compared with other breeds such as Angus (Lunt et al., 1993; Gotoh et al., 2009). As such, it is possible that a reduced number of muscle fibers are formed in Wagyu cattle because of the great number of mesenchymal progenitor cells that shifts to fibrogenic/ adipogenic lineage, leading to a greater formation of adipose and connective tissue. Consequently, it would explain the greater muscle fiber size in Wagyu muscle as they might have a reduction in the number of muscle fibers leading to their greater hypertrophy. The larger muscle fiber size found in Wagyu cattle compared with Angus cattle corroborates another report that showed that Wagyu cattle had larger muscle fibers compared with Holsteins (Albrecht et al., 2011). However, we did not observe significant difference in the expression of MyoD and MyoG, 2 key transcription factors regulating myogenesis, indicating no difference in myogenesis in steers. Nevertheless, we cannot exclude the possibility that there was difference between Wagyu and Angus cattle during the early developmental stages when myogenesis is actively ongoing. Further studies are needed to clarify whether there is difference in myogenesis between Wagyu and Angus cattle.

Wagyu Cattle had Enhanced Adipogenesis and Fibrogenesis Compared with Angus Cattle

Adipogenesis, especially the terminal differentiation of adipocytes requires the concerted action of PPAR γ and C/EBP α , which acts synergistically to induce differentiation-linked gene expression (Rosen et al., 2002). Recently, Zfp423 was demonstrated as a very early marker for adipogenesis, which induces adipogenic commitment and the upregulation of PPAR γ expression (Gupta et al., 2010, 2012). In this study we observed greater mRNA expression of Zfp423, PPAR γ , Duarte et al.



Figure 5. Immunofluorescent staining of Angus and Wagyu sternomandibularis muscle. Fatty acid binding protein (FABP4) stained green and nuclei counterstained with DAPI (4',6-diamidino-2-phenylindole) blue (A). Density of preadipocytes and adipocytes counted according to the positive staining for both FABP4 and DAPI (B) (*P < 0.05; n = 3; mean \pm SE). See online version for figure in color.

and C/EBP α in Wagyu muscle, consistent with high marbling observed in Wagyu beef. In addition, a greater expression of FABP4 was detected in spaces between muscle fibers of Wagyu compared with Angus muscle, indicating enhanced proliferation and/or adipogenic differentiation of progenitor cells in Wagyu muscle, leading to the greater density of preadipocytes and adipocytes. Because the expression of FGF-2 and its receptor was greater in Wagyu compared with Angus muscle, enhanced proliferation is expected for Wagyu progenitor cells compared with Angus muscle.

Both collagen content and its solubility are important factors contributing to meat tenderness. Intermolecular cross-links provide stability to collagen fibrils (Eyre and Wu, 2005), which increases stiffness and reduces tenderness of meat (Duarte et al., 2011). Fibrogenesis, a process leading to collagen synthesis and cross-linking, is mainly mediated by TGF- β signaling pathway (Chen et al., 2005; Salvadori et al., 2005), which promotes fibrosis via activation of the SMA- and MAD-related protein signaling pathway (Gosselin et al., 2004; Decologne et al., 2007) to induce the expression of fibrogenic genes, including fibronectin and type I collagen (Kennedy et al., 2008). In Wagyu cattle muscle, the expression of fibrogenic markers, fibronectin, type I collagen, and

TGF- β were greater when compared with Angus cattle, consistent with enhanced fibrogenesis in Wagyu cattle. In addition, we also detected greater collagen content and lower collagen solubility in Wagyu cattle. In our previous study, we observed that collagen content and cross-linking in skeletal muscle are correlated (Huang et al., 2012). The trichrome staining, which stains collagen fibers blue, shows the widespread distribution of collagen in the space between Wagyu muscle fibers. On the other hand, Angus muscle fibers appear to be tightly attached to each other and collagen is mostly located between the muscle bundles.

Possible Mechanisms Leading to Enhancement of Adipogenesis and Fibrogenesis in Wagyu Cattle

Mesenchymal fibro/adipogenic progenitor cells contribute to ectopic fat formation in skeletal muscle (Uezumi et al., 2010) and also has the ability to differentiate into fibroblasts (Joe et al., 2010). Due to the dual differentiation capacity of fibro/adipogenic progenitor cells, their differentiation can be considered as a competitive process if the proliferation of progenitor cells is unchanged. We originally hypothesized that the adipogenic differentiation is enhanced and fibrogenic differentiation is reduced in Wagyu muscle. Unexpectedly, our results show that both adipogenesis and fibrogenesis were enhanced in Wagyu muscle, which strongly suggest that the abundance of progenitor cells is enhanced in Wagyu muscle elevating both fibrogenesis and adipogenesis. Indeed, in this study, we observed that there were larger spaces between muscle fibers that were filled with extracellular matrix in Wagyu muscle, and more abundant intramuscular preadipocytes and other cells were detected.

To explain possible mechanisms leading to increased abundance of progenitor cells, we also analyzed the expression of FGF-2 and its receptor, which are known to be critical for the proliferation of progenitor cells (Suga et al., 2009; Yun et al., 2010). In this study, we observed that FGF-2 expression was greater in the muscle of Wagyu compared with Angus cattle, which was consistent with the more abundant intramuscular preadipocytes and adipogenic cells in Wagyu cattle muscle. The greater abundance of adipogenic cells in Wagyu muscle also indicates that the overall adipogenic differentiation in Wagyu was greater compared with Angus cattle muscle. Indeed, the expression of Zfp423, a transcription factor committing progenitor cells to the adipogenic lineage, was greater in Wagyu compared with Angus cattle. The mechanisms leading to the high Zfp423 expression in Wagyu compared with Angus cattle warrant further studies.

Conclusions

Both marbling and connective tissue are increased in Wagyu cattle compared with Angus cattle, and myogenesis appears to be reduced in Wagyu cattle. The enhancement of adipogenesis and fibrogenesis is likely due to the greater abundance of fibro/adipogenic progenitor cells in Wagyu cattle. In addition, the richness of adipogenic cells in Wagyu muscle indicates that adipogenic differentiation of progenitor cells is greater in Wagyu compared with Angus cattle muscle, which warrants further studies.

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