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Reduced satellite cell density and myogenesis in Wagyu compared with Angus cattle as a possible explanation of its high marbling

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Mechanisms responsible for excellent marbling in Japanese black cattle, Wagyu, remain to be established. Because both muscle cells and intramuscular adipocytes are developed from mesenchymal progenitor cells during early muscle development, we hypothesized that intramuscular progenitor cells in Wagyu cattle have attenuated myogenic capacity in favor of adipogenesis, leading to high marbling but reduced muscle growth. Biceps femoris *muscle biopsy samples were obtained from both Angus (*n *= 3) and Wagyu (*n *= 3) cattle at 12 months of age. Compared with Angus, the density of satellite cells was much lower in Wagyu muscle (by 45.8 ± 10%,* P *< 0.05). Consistently, the formation of myotubes from muscle-derived progenitor cells was also lower (by 64.2 ± 12.9%,* P *< 0.05), but adipogenic capacity was greater in Wagyu. The average muscle fiber diameter was larger in Wagyu (by 23.9 ± 6.8%,* P *= 0.089) despite less muscle mass, suggesting less muscle fiber formation in Wagyu compared with Angus cattle. Because satellite cells are derived from fetal myogenic cells, the reduction in satellite cell density together with lower muscle fiber formation suggests that myogenesis was attenuated during early muscle development in Wagyu cattle. Given the shared pool of mesenchymal progenitor cells, the attenuated myogenesis likely shifts progenitor cells to adipogenesis during early development, which may contribute to high intramuscular adipocyte formation in Wagyu cattle.*

Keywords: intramuscular lipid, myogenesis, marbling, satellite cell, skeletal muscle

Implications

Wagyu cattle are well known for high intramuscular lipid content. Results from this study shows that the satellite cell density in Wagyu cattle is lower than Angus cattle, which limits the muscle growth potential of Wagyu cattle. The attenuated muscle growth may direct nutrients to intramuscular adipose depot and contribute to high marbling in Wagyu cattle.

Introduction

The palatability of beef is correlated with the degree of marbling and tenderness, two top quality issues for the beef industry (Garcia *et al.*, 2008). Marbling lipid provides juiciness and flavor, as well as smoothness of texture due to the lubrication effect of lipid (Dryden and Maechello, 1970). Tenderness is another important factor determining consumer satisfactory of beef consumption. Tenderness is determined by the myofibrillar effect and background toughness; the background toughness correlates with collagen content and cross-linking, which increases as animals become older (Archile-Contreras *et al.*, 2010). As a result, the USDA quality grading of beef carcasses is based on marbling and maturity, and beef from young cattle (Maturity A and B, accounts for vast majority of beef carcasses in the United States) is qualified for high-quality grades. However, only 2.1% of carcasses are above the Slightly Abundant marbling necessary to qualify for Prime grade (Moore *et al.*, 2012), though the ratio of Prime grade increases in last several years (Boykin *et al.*, 2017). The selection for high lean growth, the use of implants, and harvesting at increasingly younger ages could also reduce intramuscular adipose tissue (Albrecht *et al.*, 2011; Du *et al.*, 2013). Thus, it is important to enhance marbling in beef cattle.

Skeletal muscle and intramuscular adipocytes are originated from the same pool of stem cells in paraxial mesoderm during early embryonic development, and continue to develop and grow in the same physiological environment (Gesta *et al.*, 2007; Du *et al.*, 2013). In livestock, all muscle fibers are formed before birth and postnatal muscle growth is

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due to muscle fiber hypertrophy (Brameld *et al*., 2000). Muscle satellite cells, originated from myogenic cells during fetal development, are critically important for postnatal muscle growth (Bailey *et al*., 2001). Their proliferation and fusion with existing muscle fibers contribute to the majority of nuclei in adult muscle fibers (Davis and Fiorotto, 2009). On the other hand, if myogenesis during fetal development is weakened, both the number of muscle fibers and the density of satellite cells are reduced, resulting in permanent reduction of muscle mass (Zhu *et al*., 2004 and 2008). In addition, fetal and neonatal stages are also important for adipose tissue development, and intramuscular adipogenesis during early development generates adipocytes for later lipid accumulation during fattening, which forms marbling adipose tissue (Zhu *et al*., 2008; Yan *et al*., 2010; Du *et al*., 2015).

The Japanese Wagyu cattle is well known for its extremely high marbling. In our previous study, we found that high intramuscular adipogenesis in Wagyu cattle is a major factor contributing to high marbling (Duarte *et al*., 2013). Despite the excellent intramuscular lipid accumulation, Wagyu cattle has lower growth rate and smaller muscle mass compared with Angus and other common domestic cattle breeds at comparable age (Lunt *et al*., 1993; Afolayan *et al*., 2007) , suggesting a low efficiency of myogenesis and muscle growth rate. Because myogenic cells and intramuscular adipocytes share developmental origins (Du *et al*., 2013), we hypothesized that, compared with Angus cattle, the myogenic differentiation and myogenic cells in Wagyu cattle are attenuated, which contributes to the enhanced intramuscular lipid accumulation.

Material and methods

Animals and skeletal muscle tissue sampling

Wagyu ($n = 5$, BW $= 302 \pm 9$ kg) and Angus ($n = 5$, BW $= 398 \pm 12$ kg) heifers were managed under the same condition in the Beef Center of Washington State University (WSU). At 12 months of age, skeletal muscle tissue was obtained by biopsy per the protocol approved by the Institutional Animal Care and Use Committee of Washington State University. Briefly, an area (12×12 cm) over the right *Biceps femoris* muscle of the restrained cattle was clipped, followed by surgical preparation via standard techniques using a combination of povidine iodine scrub, 75% ethanol, and povidine solution. Lidocaine (2%) was locally injected for analgesia. Then a 2-cm incision using a surgical blade was made through the skin to expose the underlying subcutaneous tissues. Using blunt-end scissors, about 1 g of muscle tissue together with subcutaneous adipose tissue was collected from each animal. Muscle samples were separated from subcutaneous adipose tissue and were further divided into three portions, with one portion snap frozen in liquid nitrogen for biochemical analyses, one embedded for cryosection, and the remaining portion submerged in PBS supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml Fungizone B and used for cell separation. Note that due to the limited amount of biopsy tissue, we were only able to obtain a sufficient number of muscle-derived cells from three cattle of each genotype for cell analyses, including myogenesis, adipogenesis, and gene expression examination. And to ensure consistency, only tissues from these six animals were used for histological analysis.

Antibodies

Anti-myogenin (F5D), anti-Pax7 (PAX7), anti-MHC (MF20) and anti-β-tubulin (E7) mouse monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). Anti-desmin (ab15200) was purchased from Abcam (Cambridge, MA, USA). Goat anti-mouse Alexa Fluor 555 (#4409) and goat anti-rat Alexa Fluor 488 (#4416) antibodies were purchased from Cell Signaling (Danvers, MA, USA).

Muscle cell isolation and culture

Muscle tissue was digested as previously described (Fu *et al*., 2013). Briefly, muscle tissue was rinsed with PBS and minced with a dissecting scissors. Minced muscle tissue was then digested in Dulbecco's Modified Eagle's medium (DMEM) contaning 0.75 U/ml collagenase D and 1 U/ml of Dispase II for 1 h at 37°C. The tissue slurry was filtered through a 100 μm cell strainer and pelleted through centrifugation. The pellet was resuspended in growth medium composed of F10 medium with 20% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml Fungizone B. Cell culture medium was changed every 2 days. Myogenic differentiation was induced by replacing the medium with differentiation medium containing DMEM with 2% horse serum. Adipogenic differentiation was induced as previously described (Wei *et al*., 2015). Briefly, cells were cultured in adipogenic differentiation medium containing DMEM + 10% FBS supplemented with 1 μg/ml insulin, 1 μM dexamethasone and 0.5 mM isobutylmethylxanthine for 6 days, followed by treatment with DMEM + 10% FBS supplemented with 1 μg/ml insulin only for 8 more days.

Real-time quantitative PCR

Total RNA was extracted using Trizol (Sigma, Saint Louis, MO, USA) followed by DNase (NEB, Ipswich, MA, USA) treatment, and cDNA was synthesized using a reverse transcription kit (Bio-Rad, Hercules, CA, USA). Real-time PCR (RT-PCR) was carried out using CFX RT-PCR detection system (Bio-Rad) with a SYBR Green RT-PCR kit from Bio-Rad. After amplification, a melting curve (0.01°C/s) was used to confirm product purity, and agarose gel electrophoresis was performed to confirm that only a single product of the right size was amplified. Relative mRNA content was normalized to 18S rRNA content (Fu *et al*., 2013). Primer sequences and their respective PCR fragment lengths are listed in Table 1.

Western blot analysis

Western blot was conducted as previously described (Fu *et al*., 2013). Immunoreactive proteins in the membrane

Table 1 *Primer sequences and their respective PCR amplicon sizes*

Genes	Amplicon sizes	Forward	Reverse
18S rRNA	118 bp	CCTGCGGCTTAATTTGACTC	AACTAAGAACGGCCATGCAC
Pax7	107 bp	CGGGCATGTTTAGCTGGGAGA	TCTGAGCACTCGGCTAATCGAAC
MyoD	111 bp	GAACTGCTACGACCGCACTTACT	GAGATGCGCTCCACGATGCT
Myf5	93 bp	CCCACCAGCCCCACCTCAAGT	GTAGACGCTGTCAAAACTGCTGCT
Myogenin	131 bp	CTCAACCAGGAGGAGCGCGAC	TTGGGGCCAAACTCCAGTGCG
ZNF423	120 bp	GGATTCCTCCGTGACAGCA	TCGTCCTCATTCCTCTCCTCT
PPARγ	111 bp	TGGAGACCGCCCAGGTTTGC	AGCTGGGAGGACTCGGGGTG

were scanned and analyzed by Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE, USA). Band density was normalized according to the β-tubulin content.

Immunocytochemical staining

Cells grown on multiple well plates were fixed in cold methanol for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, blocked with 3% BSA, and incubated with primary antibodies (anti-desmin 1 : 100; anti-MHC 1 : 50) at 4°C overnight. Cells were then stained with corresponding secondary antibodies (1 : 1000) for 1 h. Cells were then mounted in a mounting medium containing DAPI (Vector Lab, Burlingame, CA, USA). Images were taken using a EVOS microscope (Thermo Fisher Scientific, Rockford, IL, USA).

Immunohistochemical staining

The *B. femoris* muscle sample was fixed in 4% paraformaldehyde and frozen in isopentane cooled in liquid nitrogen. Frozen tissue was sectioned (10 μm thick). Sections were heated in citrate buffer for 20 min, blocked in 5% goat serum in TBS containing 0.3% Triton X-100, and stained with anti-Pax7 antibody (1 : 5) and corresponding fluorescent seconday antibody (1 : 1000). Sections were then mounted in a mounting medium containing DAPI .

Oil-Red O staining

Oil-Red O staining was performed as previously described (Wei *et al.*, 2015). Briefly, after 14 days of adipogenic differentiation cells were fixed in 10% formalin for 30 min and stained with Oil-Red O in 60% isopropanol for 10 min.

Quantification of satellite cells

$Pax7^+$ cells with nuclei identified by DAPI staining were classified as satellite cells. For each muscle sample, satellite cells in four randomly picked microscopic fields of each of three sections at different depths of each muscle were counted (four fields/section, three sections/muscle sample). The sums of satellite cells in the four picked fields of each section were caculated. Average numbers obtained from the three examined sections of each muscle sample were used as a biological replicate for comparative analysis.

Minimum muscle fiber diameter measurement

Gill's Hematoxylin (26030-10) and Eosin Y–Phloxine B (26051-21) were purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Hemotoxylin and eosin staining to frozen sections was conducted following the manufacturer's protocol. The smallest diameter of muscle fibers was measured using Fiji Image J as described previously (Duarte *et al.*, 2013).

Statistical analysis

All experiments were performed using three animals per breed. Due to the large difference in marbling and myogenic characteristics between Wagyu and Angus cattle, three animals per breed should provide power to discern biological effects (Whitley and Ball, 2002; Duarte *et al.*, 2013). Each individual animal is an experimental unit. Statistical analysis was performed using SAS 9.2 (Statistical Analysis System Institute Inc., Cary, NC, USA) and means ± SEM were reported. A bilateral *t*-test was used to identify difference and statistical significances were considered at $P < 0.05$. *Post-hoc* power analysis was conducted for each experiment and the power to discern genotype effect is shown in the corresponding figure legend.

Results

Satellite cell density was lower in Wagyu than that in Angus muscle

Muscle sections were staining with anti-Pax7 antibody for satellite cell identification. Satellite cell density was significantly lower in Wagyu than in Angus muscle (Figure 1a). Consistently, the mRNA level of Pax7 was lower in Wagyu muscle (Figure 1b). To better understand the single nucleated cell composition of muscle from Wagyu and Angus, these cells were isolated from muscle through enzymatic digestion. Two days after cell isolation, cells were stained with an antibody against desmin, a myoblast marker. As expected, less cells were stained positive for desmin in Wagyu as compared with Angus cells (Figure 2a). Moreover, Myf5 and MyoD, two other myoblast markers were expressed at lower levels in cells isolated from Wagyu than cells from Angus muscle, further indicating reduced myogenic cell density in Wagyu muscle compared with Angus (Figure 2b).

Myogenic differentiation of muscle-derived cells isolated from Wagyu was lower than that from Angus

To compare the myogenic capacity of muscle-derived cells isolated from Wagyu and Angus, these cells were induced for

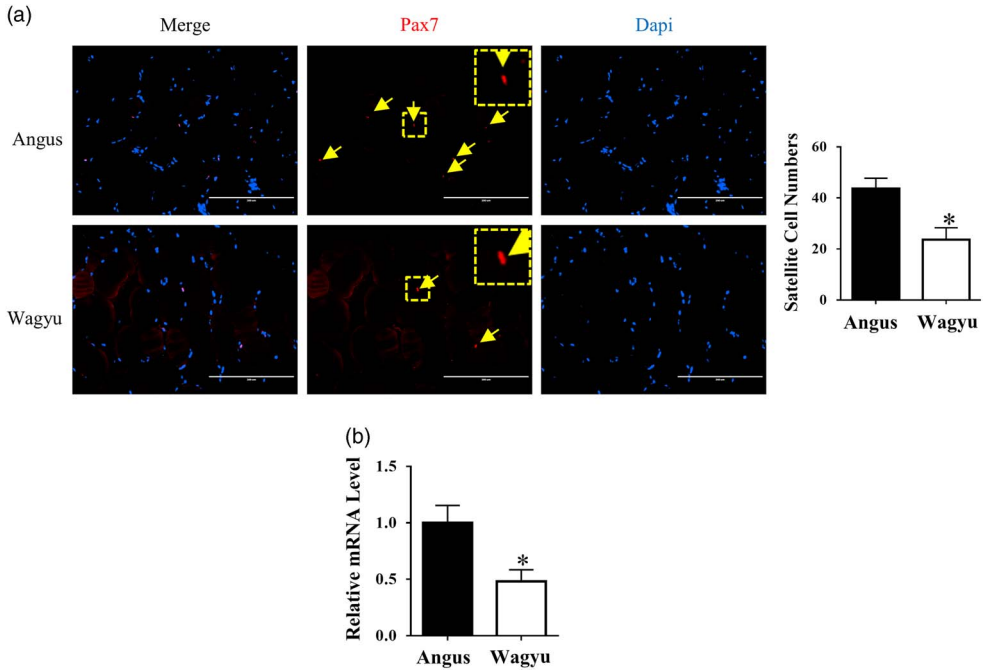

Figure 1 Satellite cell density in Wagyu cattle was lower than Angus cattle. (a) Muscle sections were stained with anti-Pax7 antibody to quantify the abundance of satellite cells (pointed by arrows) (Power = 0.9). (b) Pax7 mRNA expression level in muscle of Angus cattle and Wagyu cattle was measured by real-time PCR (Power = 0.8). $^*P < 0.05$; *v.* Angus; mean ± SEM; $n = 3$. Scale bars = 200 μm.

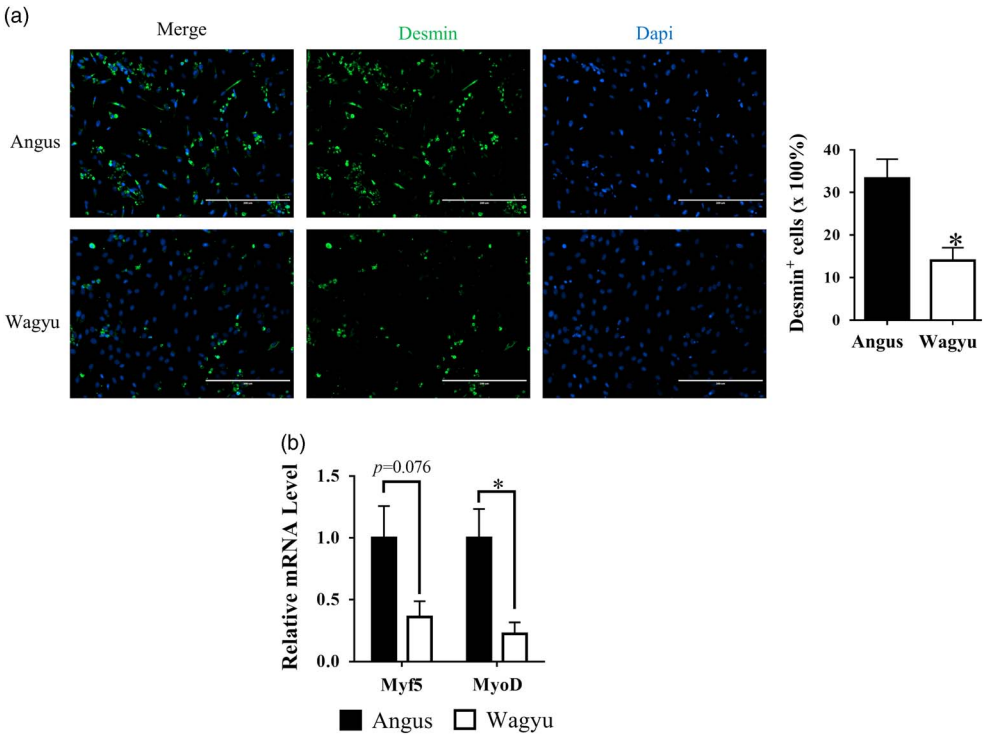

Figure 2 Muscle-derived cells from Wagyu cattle contained less activated myoblasts than Angus cattle. Muscle-derived cells were isolated from Angus and Wagyu cattle, and were cultured in growth medium. (a) Cultured muscle-derived cells were stained with anti-desmin antibody to quantify activated myoblasts (Power = 0.95). (b) The mRNA expression levels of myoblast markers, Myf5 (Power = 0.66) and MyoD (Power = 0.9), in muscle-derived cells from Angus and Wagyu cattle were measured by real-time PCR. $^*P < 0.05$; *v.* Angus; mean ± SEM; $n = 3$. Scale bars = 200 μm.

myogenic differentiation. MyoD and myogenin mRNA expression levels were lower in cells from Wagyu compared with Angus after 2 days of myogenic induction (Figure 3a), consistent with significantly lower protein levels of myogenin and myosin heavy chain (MHC) in Wagyu after 4 days of myogenic induction, indicating that Wagyu cells had reduced

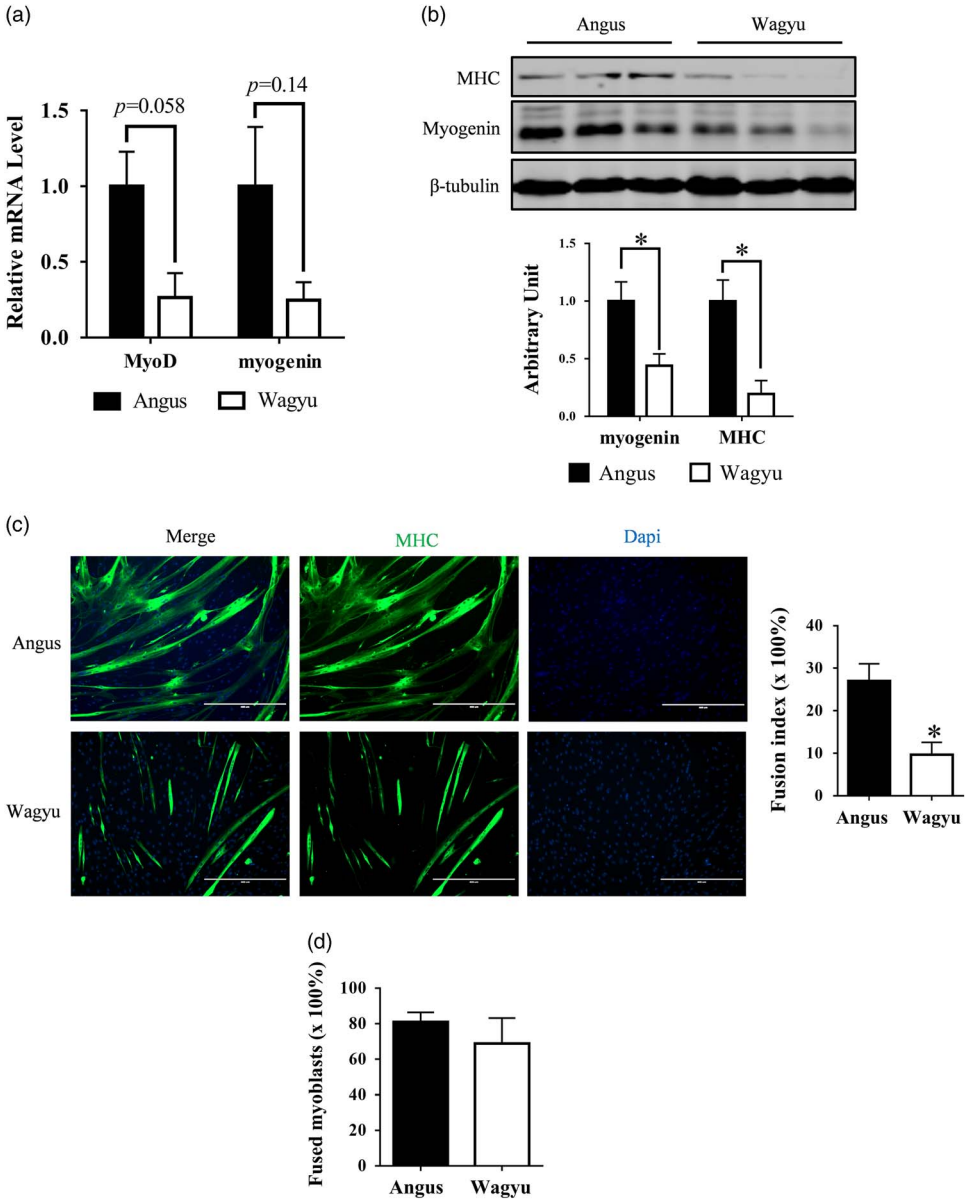

Figure 3 Myogenic differentiation of muscle-derived cells from Wagyu cattle was less than Angus cattle. Muscle-derived cells isolated from Angus and Wagyu cattle were cultured in growth medium until 80% confluency and were then switched to myogenic differentiation medium to induce myogenic differentiation. (a) The mRNA expression levels of MyoD (Power = 0.75) and myogenin (Power = 0.45) in muscle-derived cells from Angus and Wagyu cattle at 2 days after the induction of myogenic differentiation were measured by real-time PCR. (b) Protein contents of myogenin (Power = 0.81) and MHC (Power = 0.96) in muscle-derived cells from Angus and Wagyu cattle at 4 days after the induction of myogenic differentiation were measured by Western blot. (c) Immunocytochemistry staining of differentiated myotubes in muscle-derived cells from Angus and Wagyu cattle at 4 days after the induction of myogenic differentiation and calculated fusion index (Power = 0.94). (d) Normalized fusion index indicating percentage of fused myoblasts was calculated by dividing fusion index (Figure 3c) by the percentage of myoblasts in total muscle-derived cells (Figure 2a) (Power = 0.12). MHC = myosin heavy chain; $^*P < 0.05$; *v.* Angus; mean ± SEM; $n = 3$. Scale bars = 200 μm.

myogenic differentiation (Figure 3b, Supplementary Material Figure S1). Myotube formation was further quantified by MHC immunocytochemical staining. As expected, less myotubes were seen in cells from Wagyu compared with Angus (Figure 3c). However, after normalizing the fusion index by the myogenic cell density, there was no difference in the efficiency of myoblast fusion into myotubes, suggesting that the lower myogenic differentiation of muscle-derived cells from Wagyu was mainly due to reduced myoblast numbers (Figure 3d). In contrast to myogenesis, adipogenic markers, ZNF423 and PPARγ, were expressed at greater levels in cells isolated from Wagyu than cells from Angus after 2 days of adipogenic induction, showing greater adipogenesis, which might be a combined effect of a greater number of adipogenic cells and enhanced adipogenic differentiation (Figure 4a). Consistently, lipid accumulation of cells isolated from Wagyu appeared greater than cells from Angus after 14 days of adipogenic induction (Figure 4b).

Average muscle fiber diameter was larger in Wagyu compared with Angus muscle

To analyze the impact of reduced satellite cells on muscle growth in Wagyu cattle, we measured the diameter of muscle fibers of Wagyu and Angus cattle. Surprisingly, the average diameters of muscle fibers from Wagyu were larger than those of Angus (by 23.9 ± 6.8%, $P = 0.089$), indicating that there was a smaller number of muscle fibers in Wagyu cattle compared with Angus cattle, suggesting that limited myogenesis in Wagyu muscle may contribute to their propensity to deposit more marbling (Figure 5).

Discussion

In livestock, muscle fibers are formed before birth through two separated stages of myogenesis including primary myogenesis during the embryonic stage and secondary myogenesis primarily during the second trimester in cattle (Bonnet *et al.*, 2010). Enhanced proliferation of myogenic progenitor cells and their myogenic differentiation during fetal development promote muscle fiber formation which increases offspring lean mass (Zhu *et al.*, 2004). Genetics such as myostatin mutation, maternal nutrition and physiological conditions affects fetal myogenesis and the subsequent formation of muscle fibers (Tong *et al.*, 2009; Yan *et al.*, 2010). In addition, during fetal development, a portion of myogenic cells become quiescent, which locate within basal lamina and form satellite cells in postnatal muscle. Thus, myogenic activity during the fetal stage correlates with satellite cell density in mature muscle (Bailey *et al.*, 2001).

Wagyu cattle are well known for producing high-quality beef with extremely high marbling (Lunt *et al.*, 1993; Zembayashi and Lunt, 1995). Previously, we observed that both adipogenesis and fibrogenesis are greater in Wagyu compared with Angus cattle (Duarte *et al.*, 2013). Because myogenic and fibro/adipogenic cells share a common pool of progenitor cells in early embryo (Du *et al.*, 2015), the greater adipogenic differentiation in Wagyu cattle is postulated to reduce myogenic differentiation. However, the muscle fiber formation and myogenic cells of Wagyu cattle has not been characterized. Satellite cell fusion into existing muscle fibers is a major mechanism of postnatal muscle growth (Davis and Fiorotto, 2009). Thus, satellite cell density was analyzed by immunostaining for Pax7, a marker of satellite cells (Davis and Fiorotto, 2009). We found that the satellite cell density in Wagyu cattle is significantly lower than that of Angus cattle, suggesting attenuated myogenesis during early development, considering that satellite cells are descendants of myogenic cells during fetal muscle development. Consistently, Wagyu muscle contains less muscle fibers than Angus muscle (Duarte *et al.*, 2013). Given that muscle fibers are formed during early development, less muscle fibers further confirms the lower myogenesis occurred in Wagyu compared with Angus cattle. The lower myogenesis might not be due to impairment in myogenic differentiation of satellite cells, but rather the reduction in the abundance of satellite cells in overall muscle-derived single nuclear cells, a

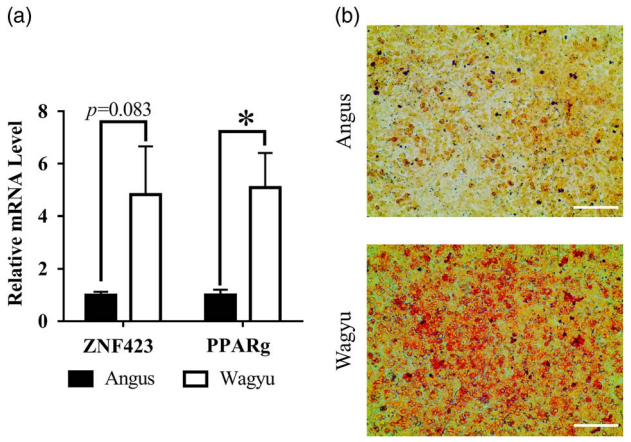

Figure 4 Adipogenic potential of muscle-derived cells from Wagyu cattle was greater than cells from Angus cattle. Muscle-derived cells isolated from Angus and Wagyu cattle were cultured in growth medium until 80% confluency and were then switched to adipogenic differentiation medium to induce adipogenic differentiation. (a) The mRNA expression levels of ZNF423 (Power = 0.63) and PPARγ (Power = 0.86) in muscle-derived cells from Angus and Wagyu cattle at 2 days after the induction of adipogenic differentiation were measured by real-time PCR. (b) Representative images of Oil-Red O staining performed at 14 days of adipogenic differentiation. $^*P < 0.05$; *v.* Angus; mean ± SEM; $n = 3$. Scale bar = 400 μm.

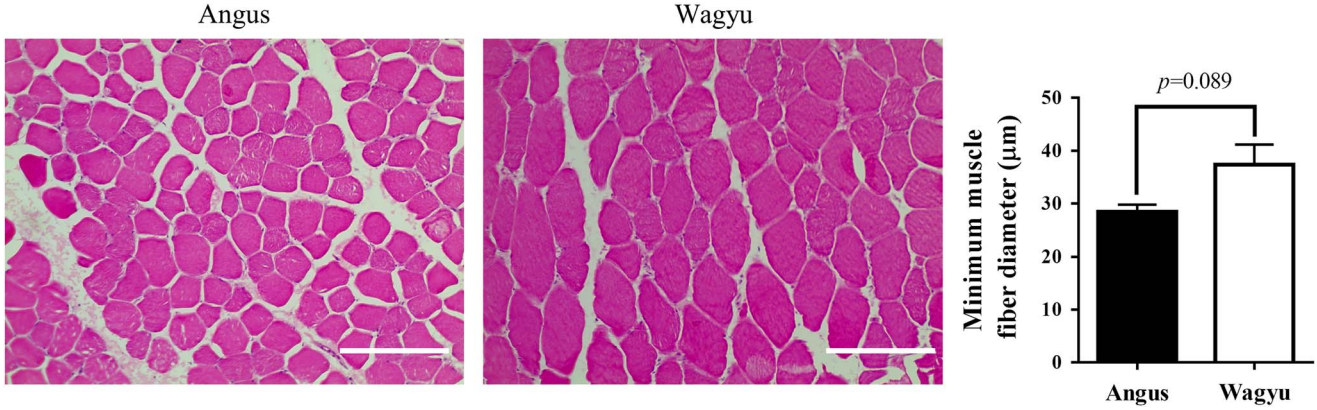

Figure 5 Muscle fiber size of Wagyu cattle was larger than Angus cattle. HE staining was performed to muscle sections to analyze the minimum muscle fiber diameters of Angus and Wagyu cattle (Power = 0.61). HE = hemotoxylin and eosin; mean ± SEM; $n = 3$. Scale bars = 200 μm.

notion supported by the greater adipogenic capacity of Wagyu muscle-derived cells. Very interestingly, muscle fibers of Wagyu cattle were found to be larger than those of Angus cattle, which could be due to a compensatory hypertrophy of existing muscle fibers during muscle growth. Wagyu cattle grow slower than Angus cattle and, thus, require longer feeding to achieve the same market weight of Angus, resulting in compensatory muscle fiber hypertrophy and similar muscle cross area of Wagyu and Angus cattle at harvest (Lunt *et al*., 1993). In addition, Wagyu muscle has much higher intramuscular lipid and collagen content, further supporting the smaller muscle fiber number in Wagyu cattle (Duarte *et al*., 2013). In combination, these data point to a shift from myogenesis to adipo/fibrogenesis during early embryonic development in Wagyu cattle. This notion is consistent with observation in runt piglets; compared with littermates, runt piglets do not obtain sufficient nutrients during fetal development, which impair muscle fiber formation and have less muscle mass permanently (Aberle, 1984), but the marbling and overall adiposity are greater (Bushman, 2007). However, the underlying mechanisms leading to such a shift in Wagyu cattle remain to be established.

Of note, one possible limit of this study is that only *B. femoris* was tested and the physiology of different muscles may differ from each other. However, the high intramuscular fat content is constantly observed in meats derived from various Wagyu muscles compared with Angus muscles, which suggests that our findings could be extended to other muscles as well, including the economically important *Longissimus dorsi* muscle.

In summary, we found that Wagyu muscle has reduced satellite cell population and muscle fiber numbers compared with Angus cattle. Considering that both muscle fibers and satellite cells are results of fetal myogenesis, and the population of adipogenic cells is greater in Wagyu cattle, these data strongly suggest that the myogenesis is attenuated while adipogenic differentiation is enhanced during the early development of Wagyu cattle, which might provide an explanation to the high marbling but lower muscle mass in Wagyu compared with Angus cattle.

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Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731117002403

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