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Enhanced mitogenesis in stromal vascular cells derived from subcutaneous adipose tissue of Wagyu compared with those of Angus cattle[1]

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ABSTRACT: Japanese Wagyu cattle are well known for their extremely high marbling and lower subcutaneous adipose tissue compared with Angus cattle. However, mechanisms for differences in adipose deposition are unknown. The objective of this paper was to evaluate breed differences in the structure of subcutaneous adipose tissue, adipogenesis, and mitogenesis of stromal vascular (SV) cells between Wagyu and Angus cattle. Subcutaneous biopsy samples were obtained from 5 Wagyu (BW = 302 ± 9 kg) and 5 Angus (BW = 398 ± 12 kg) heifers at 12 mo of age, and samples were divided into 3 pieces for histological examination, biochemical analysis, and harvest of SV cells. Adipogenesis of SV cells was assessed by the expression of adipogenic markers and Oil Red-O staining, while mitogenesis was evaluated by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium dromide) test, phosphorylation of extracellular signal-regulated kinase (ERK) and protein kinase B (PKB; AKT). Based on histological analysis, Wagyu had larger adipocytes compared with Angus. At the tissue level, protein expression of peroxisome proliferator-activated receptor γ (PPARG) in Wagyu was much lower compared with that of Angus. Similarly, a lower mRNA expression of PPARG was found in Wagyu SV cells. No significant difference was observed for the zinc finger protein 423 (ZNF423) expression between Wagyu and Angus. As assessed by Oil Red-O staining, Wagyu SV cells possessed a notable trend of lower adipogenic capability. Interestingly, higher mitogenic ability was discovered in Wagyu SV cells, which was associated with an elevated phosphorylation of ERK1/2. There was no difference in AKT phosphorylation of SV cells between Wagyu and Angus. Moreover, exogenous fibroblast growth factor 2 (FGF2) enhanced mitogenesis and ERK1/2 phosphorylation of SV cells to a greater degree in Angus compared with that in Wagyu. Expression of transforming growth factor β 3 (TGFB3) and bone morphogenetic protein 2 (BMP2) in Wagyu SV cells was lower than that of Angus, providing potential clues for breed differences on proliferation of SV cells in these two cattle breeds. The results of this study suggest that subcutaneous adipose-derived SV cells of Wagyu possess a lower trend of adipogenesis but higher mitogenesis compared with those of Angus.

Key words: adipocyte, differentiation, ERK1/2, proliferation, TGFB3, Wagyu

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INTRODUCTION

Wagyu cattle possess high intramuscular fat (**IMF**; marbling) but also have lower subcutaneous fat accumulation compared with other beef cattle breeds (i.e., Angus; Lunt et al., 1993; Xie et al., 1996; Mir et al., 1999; Wheeler et al., 2004; Radunz et al., 2009). In beef cattle, IMF contributes to beef flavor and juiciness, and marbling is one of the most important economical traits for beef (Hausman et al., 2009; Du et al., 2010). Contrarily, subcutaneous fat possesses very low commercial value (Cianzio et al., 1985) and excessive subcutaneous fat accumulation decreases production efficiency (Du et al., 2013). Previous studies have provided substantial information regarding breed differences between Wagyu and other cattle breeds in their growth performance and meat quality. However, mechanisms leading to the greater marbling and lower fat accumulation in subcutaneous adipose depot of Wagyu remain unclear.

Adipose-derived stromal vascular (**SV**) cells contain abundant stem cells and preadipocytes, which comprise the progenitor pool of adipocytes (Gimble et al., 2007). Stromal vascular cell culture combined with molecular biology investigations have provided insights into mechanisms regulating fat deposition (Hausman et al., 2009). In vivo, hypertrophy of adipocytes (adipogenesis, i.e., triglyceride accumulation) and hyperplasia of adipose progenitor cells (mitogenesis) are positively correlated with increased adiposity (i.e., subcutaneous adipose tissue accumulation; Jo et al., 2009). As adipogenesis and mitogenesis are two main aspects of adipocyte development, we hypothesized that adipogenic/mitogenic capability of subcutaneous adipose-derived SV cells is different between Wagyu and Angus, which may lead to distinct fat deposition characteristics in these two cattle breeds. The objective of this study was to examine breed differences of adipogenesis and mitogenesis of SV cells between Wagyu and Angus. In addition, differentially expressed genes during SV cell development between Wagyu and Angus were also investigated in this study.

MATERIALS AND METHODS

Animals and Adipose Tissue Sampling

Wagyu (n = 5, BW = 302 ± 9 kg) and Angus (n = 5, BW = 398 ± 12 kg) heifers of the same age (12 mo) were managed under the same condition at the Beef Center of Washington State University (**WSU**), and subcutaneous adipose tissue was obtained by biopsy around 2 h after the meal in the morning. The biopsy was done consistently at the same time of the day between the two breeds. The Institutional Animal Care and Use Committee of WSU approved the use of animals in this research. For biopsy, an area (12 × 12 cm) over the right hind leg 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, 0.2~0.5 g subcutaneous adipose tissue together with muscle tissue was collected from each animal, and the muscle tissue was used for a separate study. Then, the skin incision over the biopsy site was closed by stitching. The subcutaneous adipose tissue samples of each cattle were divided into 3 pieces. One piece (> 0.3 g) was put into a pre-cold PBS (phosphate buffered saline; 1.06 mM KH_2PO_4, 155.17 mM NaCl, and 2.97 mM Na_2HPO_4–$7H_2O$, pH 7.4) containing tube and then was taken to the cell culture laboratory immediately (kept on ice). Around 0.1~0.2 g adipose tissue of each sample was placed into a cryogenic vial and then stored in liquid nitrogen for reverse transcription real-time quantitative PCR (**RT-qPCR**) and western blot analyses. Another small portion (~0.1 mg) of each sample was fixed in 4% fresh paraformaldehyde for histological analysis. Note that due to the limited amount of subcutaneous tissue available for Wagyu cattle, we were only able to obtain a sufficient number of stromal vascular cells from 3 Wagyu cattle for SV cell analyses, including adipogenesis, mitogenesis, and gene expression examination. PBS and DMEM (Dulbecco's Modified Eagle's Medium; low glucose; Hyclone, Logan, UT) used in this study were supplemented with 100 IU/ml penicillin (Gibco, Grand Island, NY), 100 μg/ml streptomycin (Gibco), and 250 ng/ml Fungizone B (Gibco).

Histological Analysis

Fixed adipose tissues were dehydrated and embedded in paraffin. Then adipose tissues were sectioned (5 mm), deparaffinized, rehydrated, and stained with hematoxylin and eosin (**H&E**; Wang et al., 2014). Adipocyte number and diameter were quantitated using Image-Pro Plus image analysis software (National Institute of Health, Baltimore, MD), and at least 400 adipocytes per animal were measured (8 images per section and 5 sections at 50 mm intervals per sample).

SV Cell Isolation and Culture

The isolation and culture of SV cells were performed as previously described with minor modifications (Huang et al., 2012). Briefly, adipose tissue was rinsed with PBS and minced with a dissecting scissor and then transferred to collagenase D (0.75 U/ml; Roche,

Mannheim, Germany) and Dipase (1.0 U/ml; Roche) containing tubes. The mixture was digested for 1 h at 37°C with constant agitation. Then, the mixture was filtered through a 100-μm and then a 40-μm cell strainer, and filtrate was centrifuged at 400 g for 5 min. The pellet containing SV cells was resuspended in DMEM +10% FBS (Fetal Bovine Serum; Gibco) media and incubated at 37°C in 5% CO_2. Cells from each sample were subcultured and then stored in liquid nitrogen for further use. Only the second passage (PN2) of these cells was used for experiments. All cell culture experiments were run in triplicate.

Adipocyte Differentiation

Both Wagyu and Angus SV cells (PN2) were seeded in two 24-well plates with 21,000 cells/cm^2. After 24 h, a standard DMI (inductive differentiation medium) cocktail (DMEM + 10% FBS supplemented with insulin [1 μg/ml], dexamethasone [1μM], and isobutylmethylxanthine [0.5 mM]) was applied to each adipogenic treatment group and kept for 6 d, followed by treatment with insulin only (1 μg/ml) for 8 more days. The control group was cultured with DMEM + 10% FBS only, and all media were changed every 2 or 3 d. At d 14 of adipocyte differentiation, cells were stained with Oil Red-O (Kinkel et al., 2004) and photographed with a microscope. To quantify lipid droplets, Oil Red-O dye retained in adipocytes was extracted with isopropanol (Sigma) and transferred to a 96-well plate to measure the optical absorbance at 510 nm by a microplate reader (Yang et al., 2013). Furthermore, Wagyu and Angus SV cells (PN2) were seeded in additional four 12-well plates with 21,000 cells/cm^2. After 24 h, RNA and protein were collected from 2 of these plates with TRIzol reagent (Invitrogen, Carlsbad, CA) to investigate gene expression without adipogenic differentiation; the other two plates were treated with the standard DMI cocktail, with RNA and protein collected via TRIzol 6 d after induction of adipogenesis to analyze the expression of adipogenic genes.

Cell Proliferation

SV cells (PN2) from Wagyu and Angus cattle were seeded in a 96-well plate with 3,000 cells/cm^2 to assess cell proliferation. After 36 h, the proliferation of SV cells was assessed using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay kit (Cayman Chemical, Ann Arbor, MI). Quantification of the cell proliferation was assessed by absorbance measurement at 570 nm. In addition, fibroblast growth factor 2 (**FGF2**; 10 ng/ml; R&D Systems, Minneapolis, MN) was further used as a mitogenic regulator to stimulate cell proliferation, followed by MTT cell proliferation assay. Briefly, SV cells (PN2) from Wagyu and Angus were seeded in several 6-well plates with 10,500 cells/cm^2 and separated into FGF2 treatment group and control (basal level) group. After 24 h, FGF2 (10 ng/ml) was added into the treatment group for 1 h; Then cells were collected for immunoblotting analyses to test extracellular signal-regulated kinase (**ERK**) and protein kinase B (**PKB**; **AKT**) activity as previously described (Zhu et al., 2009).

Real-Time Quantitative PCR

Total RNA was extracted from adipose tissue samples (0.1 g per cattle) or cell cultures (8×10^4 cells) with TRIzol reagent, followed by DNase (NEB, Ipswich, MA) treatment to remove genomic DNA. Then 1 mg RNA (A260/280 > 1.8) was reverse transcribed into cDNA using an iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA). RT-qPCR (15 μL volume per reaction) was performed with the CFX RT-qPCR detection system (Bio-Rad) using the SsoAdvanced™ SYBR Green Supermix (Bio-Rad). The following PCR conditions were used: 95°C for 3 min and 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C 30 s. Primers were designed via Primer-BLAST (NCBI), and primer sequence and amplification efficiency (**AE**) are shown in Table 1. Dissociation (melting) curves (0.01°C/s) were used to check the specificity of amplification, followed by agarose gel electrophoresis to confirm that only a single product of the correct molecular weight was amplified. 18S rRNA was used as the reference gene. The validity of 18S rRNA as a reference gene was further verified using glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) and β-catenin. RT-qPCR data were analyzed using the Praffi method to compare the relative quantity (**RQ**; fold difference) of target genes ($RQ = (1+AE)_{\text{target gene}}^{\Delta CT\ \text{target gene}} / (1+AE)_{\text{control gene}}^{\Delta CT\ \text{control gene}}$).

Western Blot Analysis

Proteins from adipose tissue samples (0.1 g per cattle) and adipogenic differentiated cells (8×10^4 cells) were extracted with TRIzol reagent. Proteins of FGF2-treated cells (1×10^5 cells) were collected with cell lysis buffer (1% SDS, 10 mmol/L Tris-HCl, pH 8.0, 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, 0.5% NP-40, and 10 mmol/L NaF) containing 10 μL of protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL) per milliliter of lysis buffer. Western blotting was performed as previously described (Yan et al., 2013). Briefly, equal amounts (30 mg) of proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad), blocked with TBS containing 5% milk for 1 h, and then immunoblotted with primary

Table 1. Primer sequences for RT-qPCR

Gene	Accession number	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Amplification efficiency
PPARG	NM_181024.2	TGGAGACCGCCCAGGTTTGC	AGCTGGGAGGACTCGGGGTG	103.84%
ZNF423	NM_001101893	GGATTCCTCCGTGACAGCA	TCGTCCTCATTCCTCTCCTCT	95.16%
EGFR	XM_002696890.1	ACTTCAGTCGTGGCAAGGAG	GGGCGCACTTTACACAGTTG	98.22%
VEGFA	NM_174216.1	CAAACCTCACCAAAGCCAGC	ACGCGAGTCTGTGTTTTTGC	104.51%
PDGFRA	NM_001192345.1	CATTGGGCACTCCTGGTCTT	ACTGCCAGCTCACTTCACTC	98.34%
HGF	NM_001031751.1	GCAAGAAAACGATGCCTCTGG	CCCGATAGCTCGAAGGCAAA	86.43%
BMP2	NM_001099141.1	TCCTGAGCGAGTTCGAGTTG	GAATCGCCGGGTTGTTTTCC	100.21%
TGFB1	NM_001166068.1	AACCTGTGTTGCTCTCTCGG	GAGGTAGCGCCAGGAATTGT	101.96%
TGFB2	NM_001113252.1	CATCTGGTCACGGTCGCGC	GGGACCTCCTCGGGTTCGGG	111.30%
TGFB3	NM_001101183.1	CTGTGCGTGAATGGCTCTTG	CATCATCGCTGTCCACACCT	102.30%
FGF2	NM_174056.3	GAAGAGCGACCCACACATCA	ACGGTTTGCACACACTCCTT	98.00%
FGF7	NM_001193131.1	CCCCGAGCGACATACAAGAAG	CAGCCACTGTCCTGATTTCCA	106.73%
FGF10	NM_001206326.1	CATTGTGCCTCAGCCTTTCC	GTGACAGGGACGGAAGACAC	104.64%
FGFR1	NM_001110207.1	AGGAGGATCGAGCCCACGGC	CTTGCTCCGGCAAGGTCGGGG	101.91%
LOX-PP	NM_173932.4	TTCCAAGCTGGCTACTCGAC	GTATGTGGACGCCTGGATGT	97.02%
COL1A1	NM_001034039.2	CCACCCCAGCCGCAAAGAGT	ACGCAGGTGACTGGTGGGATGTC	103.59%
COL3A1	NM_001076831.1	GGCCCCCTGGAAAGGACGGA	CCCCGCCAGCACCACAACAT	103.91%
FN1	NM_001163778.1	GCGTGTCACCTGGGCTCCAC	CGGTGCCGGGCAGGAGATT	108.51%
GAPDH	NM_001034034.2	TGCCCGTTCGACAGATAGCC	GCGACGATGTCCACTTTGCC	98.29%
β-*catenin*	NM_001076141.1	CGGCTTTCGGTTGAGCTGAC	GCCGTACCCACCAGAGTGAA	100.75%
18S rRNA	NR_036642	CCTGCGGCTTAATTTGACTC	AACTAAGAACGGCCATGCAC	107.07%

antibodies (1:1,000) overnight at 4°C followed by secondary antibodies (1:10,000; LI-COR) for 1 h at room temperature. The target protein bands were visualized by the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Primary antibodies against tubulin, β-actin, ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), AKT, phospho-AKT (Ser473), and peroxisome proliferator-activated receptor γ (**PPARG**) were purchased from Cell Signaling (Danvers, MA), while primary antibody against zinc finger protein 423 (**ZNF423**) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein band density was quantified and normalized to the tubulin or β-actin content.

Statistical Analysis

All experiments were performed using at least 3 animals per breed with triplicate measurements for each animal sample. Statistical analysis was performed using Statistical Product and Service Solutions (**SPSS**) 13.0. All data were presented as the mean ± SEM. Data were checked for normal distribution using QQ plots and the Shapiro–Wilk test as part of the EXPLORE procedure of SPSS. Data that were not normally distributed were logarithmically transformed before analysis. The two-tailed *t* test was used to analyze breed differences between Wagyu and Angus. For data that were still not normally distributed after transformation, the Mann–Whitney U test was used to compare differences between groups. P values less than 0.05 and P values less than 0.01 were considered statistically significant and very significant, respectively. P values less than 0.1 were considered as showing a trend for difference.

RESULTS

Histological Analysis of Adipose Tissue

Adipose tissue sections were processed for H&E staining. Results showed intercellular spaces (0~10.08 mm) existed among Wagyu adipocytes (Fig. 1A and C), whereas in Angus adipose tissue, adipocytes were closely arranged without much intercellular space (Fig. 1B and D). On the other hand, considerable H&E stained structures assembled in spaces between adjacent adipocytes of Wagyu cattle (Fig. 1C), whereas there were no clusters of H&E stained structures between adjacent adipocytes of Angus cattle (Fig. 1D). Additionally, adipocyte size distribution shifted toward larger sizes in Wagyu compared with that of Angus (Fig. 1E). Wagyu subcutaneous fat possessed larger adipocytes compared with that of Angus cattle (Wagyu: 70.11 ± 5.24 mm; Angus: 49.13 ± 3.10 mm; $P < 0.05$).

Adipogenesis of SV Cells from Wagyu and Angus Cattle

Due to the limited amount of adipose tissue (about 0.2 g) obtained from 2 Wagyu, we were only able to culture a sufficient amount of SV cells from 3 Wagyu cattle. Therefore, only 3 Wagyu SV cells were used for adipogenesis, mitogenesis, and gene expression analyses of SV cells. These SV cells from both Wagyu and

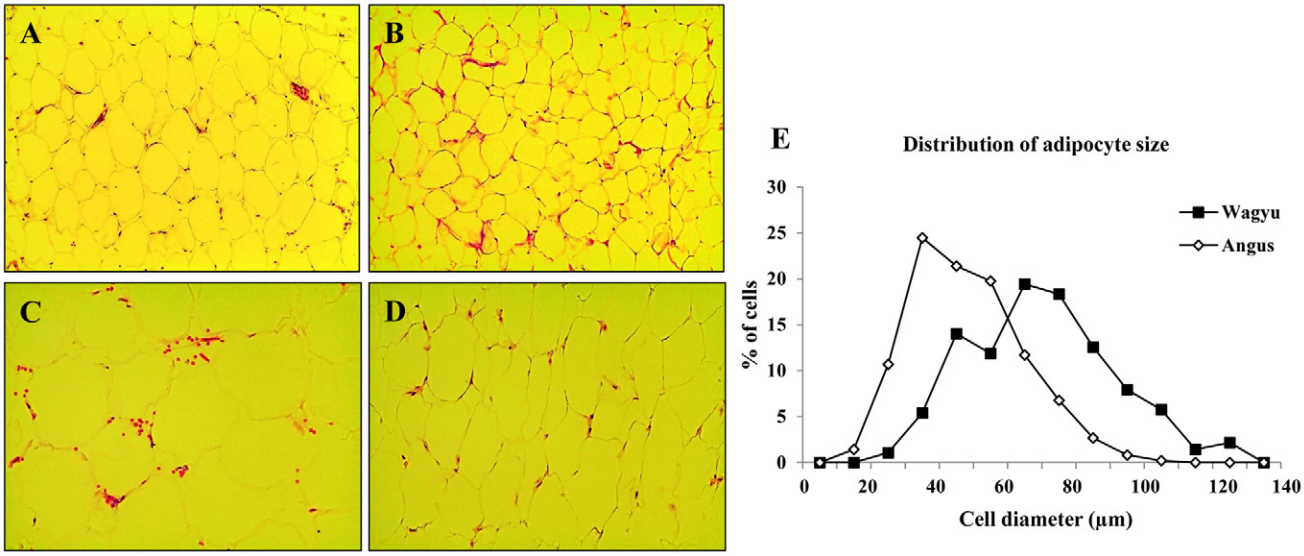

Figure 1. Histological analysis of subcutaneous adipose tissue derived from Wagyu and Angus cattle. (A, C) Sections from Wagyu cattle. (B, D) Sections from Angus cattle. (E) Distribution of adipocyte size. The data are expressed as the percentage of cells found in a given diameter size range. Images are representative of samples derived from 5 Wagyu and 5 Angus cattle. 200 × magnification in images (A) and (B); 400 × magnification in images (C) and (D).

Angus cattle were induced to adipogenic differentiation for 14 d, followed by Oil Red-O staining and quantification. As shown in Fig. 2A and B, SV cells differentiated into adipocytes after inducing for adipogenesis, while few visible adipocytes were observed in SV cells without adipogenic treatment (Fig. 2C and D). Consistent with microscopic observations (Fig. 2A and B), quantitative data on Oil Red-O staining showed a notable trend that Wagyu subcutaneous adipose-derived SV cells tended to accumulate less lipids than Angus cattle did after 14 d of adipogenic induction ($P < 0.1$; Fig. 2E). In addition, Angus SV cells expressed a higher level of PPARG both before and 6 d after adipogenic differentiation when compared with Wagyu SV cells ($P < 0.05$;

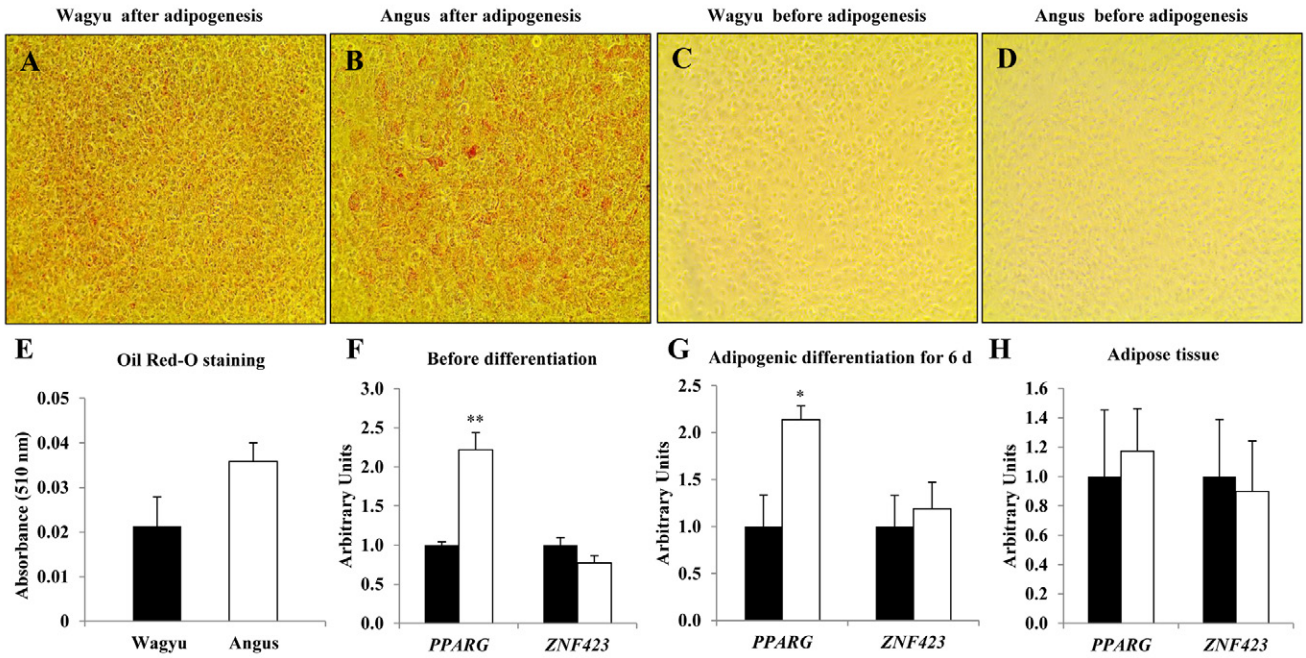

Figure 2. Adipogenic differentiation of SV cells and subcutaneous adipose tissue derived from Wagyu and Angus cattle. (A) Wagyu SV cells with adipogenic treatment. (B) Angus SV cells with adipogenic treatment. (C) Wagyu SV cells without adipogenic treatment. (D) Angus SV cells without adipogenic treatment. 200 × magnification in images (A)–(D). (E) Quantification of Oil Red-O staining. (F) ZNF423 and PPARG mRNA expression before adipogenic differentiation. (G) ZNF423 and PPARG mRNA expression after 6 d of adipogenic differentiation. (H) ZNF423 and PPARG mRNA expression in adipose tissue. The relative mRNA content of each target gene between Wagyu and Angus samples was calculated, and then, data for both Wagyu and Angus, as well as their SEM, were normalized by dividing the Wagyu mean. Images (A)–(G) are representative of samples derived from 3 Wagyu and 5 Angus cattle, while image (H) is representative of adipose tissue derived from 5 Wagyu and 5 Angus cattle. (Mean ± SEM; *$P < 0.05$; **$P < 0.01$; ■ Wagyu; □ Angus.)

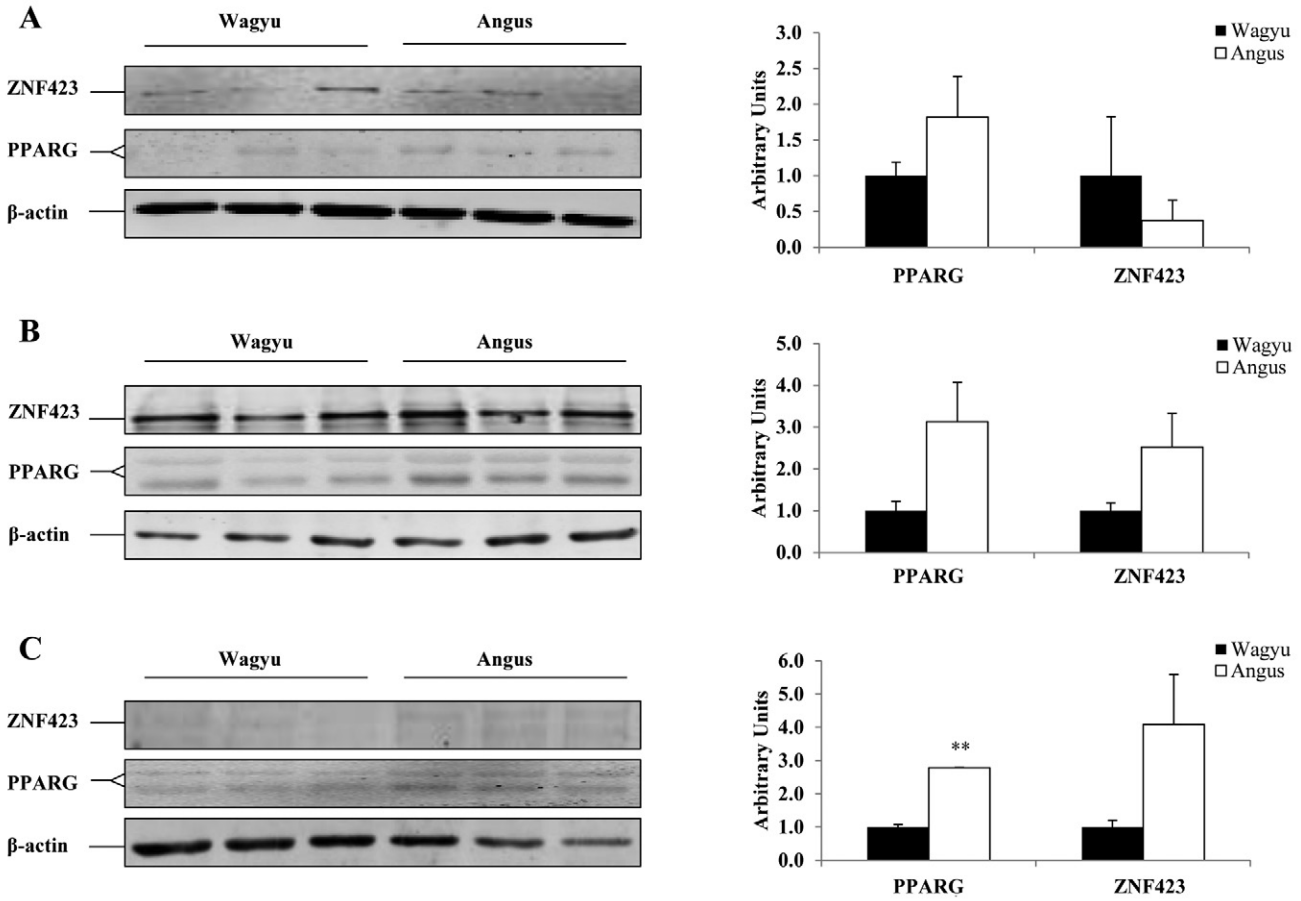

Figure 3. The protein contents of ZNF423 and PPARG in SV cells and adipose tissue derived from Wagyu and Angus cattle. (A) ZNF423 and PPARG content before adipogenic differentiation. (B) ZNF423 and PPARG content after 6 d of adipogenic differentiation. (C) ZNF423 and PPARG content in adipose tissue. The relative protein content of each target gene between Wagyu and Angus samples was calculated, and then, data for both Wagyu and Angus samples, as well as their SEM, were normalized by dividing the Wagyu mean. Images (A) and (B) are representative of adipose samples derived from 3 Wagyu and 5 Angus cattle, while image (C) is representative of adipose tissues derived from 5 Wagyu and 5 Angus cattle. (Mean ± SEM; $^{**}P < 0.01$.)

Fig. 2F and G), while no difference was observed for the expression of ZNF423 ($P > 0.05$; Fig. 2F and G). However, in adipose tissue (Fig. 2H), no significant difference was observed for the PPARG and ZNF423 mRNA expression between Wagyu and Angus cattle.

Consistent with mRNA expression levels, no difference in ZNF423 protein content was observed in both SV cells and adipose tissue ($P > 0.05$; Fig. 3). However, PPARG protein content was higher in Angus adipose tissue ($P < 0.01$) but not in SV cells ($P > 0.05$; Fig. 3). Taken together, these data indicate that Wagyu subcutaneous adipose tissue and SV cells had a lower level of PPARG mRNA and protein expression compared with those of Angus.

Higher Basal Proliferation of SV Cells in Wagyu Cattle

Wagyu SV cells possessed a higher proliferative ability compared with SV cells of Angus cattle ($P < 0.05$; Fig. 4A). However, Angus SV cells demonstrated a greater response to FGF2, which resulted in 176.42 ± 16.84% increase in cell proliferation ($P < 0.01$) compared with only 71.86 ± 17.96% increase for Wagyu cells ($P > 0.05$). After FGF2 treatment, no proliferative difference was observed between Wagyu and Angus SV cells ($P > 0.05$).

Higher Basal ERK Phosphorylation but Less Responsiveness of Wagyu SV Cells to FGF2 Compared with Angus SV Cells

Compared with Angus SV cells, Wagyu SV cells possessed higher proliferative ability, which prompted us to analyze ERK1/2 and AKT activity, 2 major signaling pathways regulating cell growth and proliferation (Hostettler et al., 2009; Rodrigues et al., 2010). Without FGF2 treatment (basal status), the phosphorylation of ERK1/2 in Wagyu was higher than that in Angus SV cells ($P < 0.05$), while no difference in the phosphorylation of AKT was observed ($P > 0.05$; Fig. 4B). Following FGF2 treatment, phosphorylation of both ERK1/2 and AKT was enhanced but at a different

degree in Wagyu versus Angus SV cells. In Wagyu SV cells, FGF2 only slightly enhanced ERK1/2 phosphorylation (42.64 ± 19.35%, $P < 0.05$) without difference in AKT phosphorylation ($P > 0.05$), while in Angus SV cells, the phosphorylation of both ERK1/2 and AKT were dramatically increased by FGF2 with 210.00 ± 58.00% ($P < 0.01$) and 133.08 ± 34.42% ($P < 0.01$), respectively. These data suggest that Angus SV cells were more sensitive to FGF2 compared with those of Wagyu.

Potential Growth Factors that Affect Proliferation of SV Cells in Wagyu and Angus Cattle

An FGF2-mediated ERK pathway could play a key role in modulating SV cell proliferation in cattle. However, no difference in endogenous FGF2 or fibroblast growth factor receptor 1 (**FGFR1**) expressions of SV cells (without any treatment) were detected ($P > 0.05$; Fig. 5A), suggesting enhanced basal ERK1/2 activity in Wagyu cattle was stimulated by other signals. To find the possible reasons for enhanced proliferation and basal ERK1/2 phosphorylation in Wagyu SV cells, we selected a panel of autocrine growth factors, including the epidermal growth factor receptor (**EGFR**), vascular endothelial growth factor A (**VEGFA**), platelet-derived growth factor receptor α (**PDGFRA**), hepatocyte growth factor (**HGF**), bone morphogenetic protein 2 (**BMP2**), transforming growth factor β 1 (**TGFB1**), transforming growth factor β 2 (**TGFB2**), transforming growth factor β 3 (**TGFB3**), FGF2, fibroblast growth factor 7 (**FGF7**), and fibroblast growth factor 10 (**FGF10**; Hostettler et al., 2009; Rodrigues et al., 2010). We also analyzed the expression of FGFR1 (Lefevre et al., 2009), lysyl oxidase propeptide (**LOX-PP**; Vora et al., 2010), collagen type I α 1 (**COL1A1**; Chen and Raghunath, 2009), collagen type III α 1 (**COL3A1**; Chen and Raghunath, 2009) and fibronectin 1 (**FN1**; To and Midwood, 2011), which are related to fibrogenesis. The mRNA expression of TGFB3 and BMP2 of Wagyu SV cells was significantly lower than that of Angus SV cells by 53.65% ($P < 0.01$) and 70.58% ($P < 0.05$), respectively. No difference was observed for the expression of other factors (Fig. 5B and C).

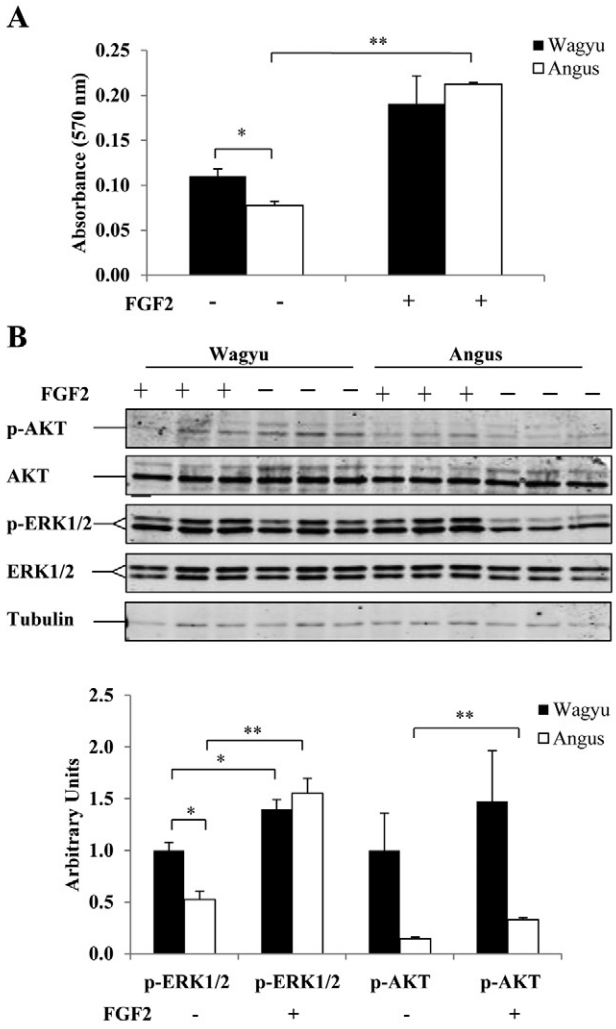

Figure 4. Cell proliferation and ERK1/2 and AKT activity of Wagyu and Angus SV cells with and without FGF2 treatment. (A) The MTT cell proliferation assay of Wagyu and Angus SV cells with and without FGF2 treatment. (B) Basal levels and FGF2 treatment on ERK1/2 and AKT activity in Wagyu and Angus SV cells. Images are representative of samples of 3 Wagyu and 5 Angus cattle. (Mean ± SEM; *$P < 0.05$; **$P < 0.01$.)

DISCUSSION

Wagyu cattle are well known for their extremely high marbling, but they have a lower subcutaneous fat content compared with that of other breeds, making Wagyu cattle one of the most commercially valuable cattle breeds. A number of studies have provided substantial comparative information between Wagyu and other cattle breeds (Lunt et al., 1993; May et al., 1993; Cameron et al., 1994; May et al., 1994; Xie et al., 1996; Wertz et al., 2002; Chung et al., 2007; Rhoades et al., 2007; Gotoh et al., 2009; Radunz et al., 2009; Yamada et al., 2009; Albrecht et al., 2011). However, physiological mechanisms leading to the lower subcutaneous fat and greater marbling of Wagyu cattle at the cellular level remain unclear.

In this study, we performed histological analyses of subcutaneous adipose tissue. Wagyu subcutaneous adipose tissue possessed larger adipocytes compared with that of Angus cattle at the age of 12 mo. This result is consistent with another report that Wagyu cattle possessed larger adipocytes compared with Holstein in intramuscular and subcutaneous adipose tissue (Albrecht et al., 2011). In addition, intercellular spaces and assembled H&E-stained structures between adjacent Wagyu adipocytes were observed, whereas in Angus adipose tissue, adipocytes were closely arranged without much intercellular space. As H&E dye could stain various cell types, the intercellular spaces

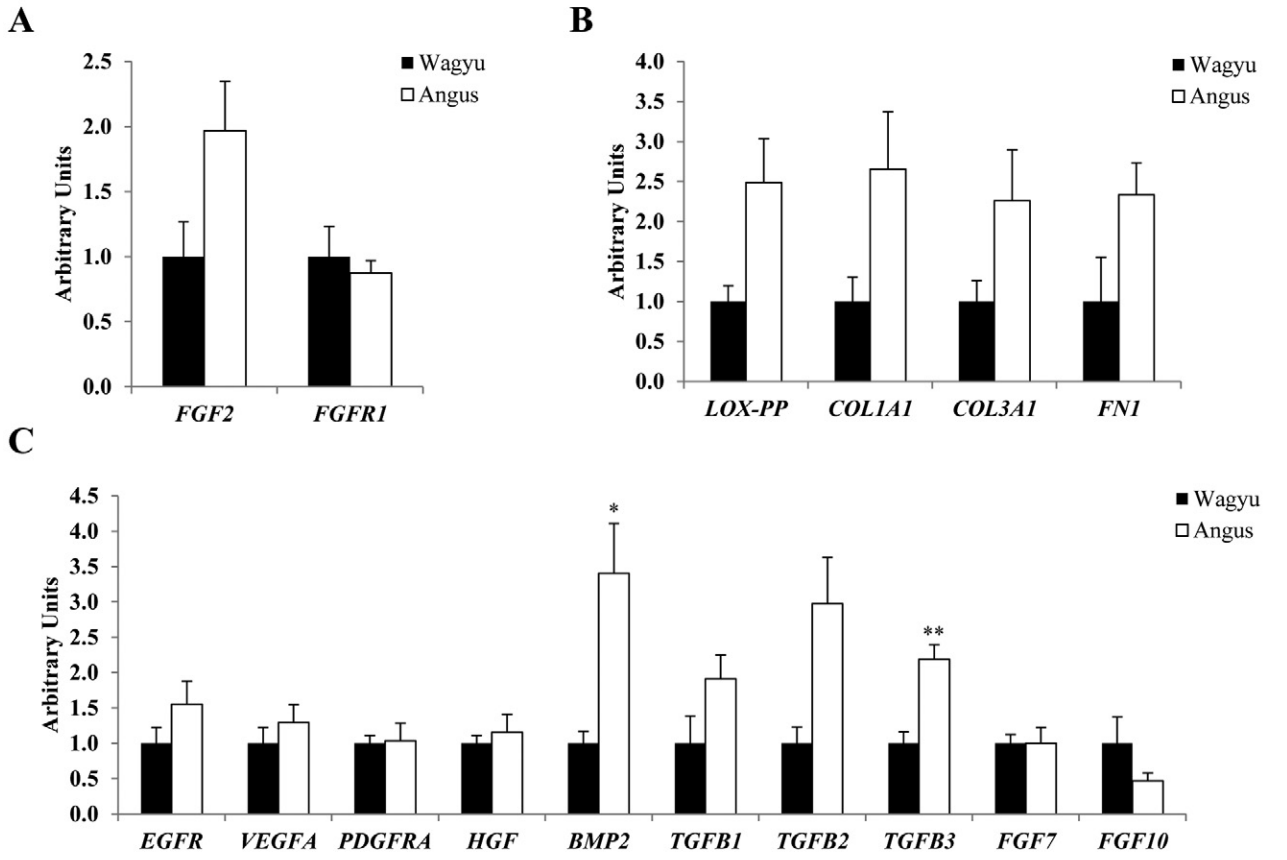

Figure 5. The mRNA expression of selected growth factors in SV cells derived from Wagyu and Angus cattle. (A) Endogenous FGF2 and FGFR1 expression. (B) Gene expression of LOX-PP, COL1A1, COL3A1, and FN1. (C) Gene expression of EGFR, VEGFA, PDGFRA, HGF, BMP2, TGFB1, TGFB2, TGFB3, FGF7, and FGF10. The relative mRNA content of each target gene between Wagyu and Angus samples was calculated, and then, data for both Wagyu and Angus samples, as well as their SEM, were normalized by dividing the Wagyu mean. Images are representative of samples derived from 3 Wagyu and 5 Angus cattle. (Mean ± SEM; *$P < 0.05$; ** $P < 0.01$.)

and H&E-stained structures among Wagyu adipocytes suggest that a large number of non-lipid-containing cells (e.g., preadipocytes and adipose-derived stem cells) may exist among Wagyu adipocytes.

By real-time qPCR and western blot assessment, it was found that Wagyu cattle possessed lower mRNA expression of PPARG in SV cell samples and lower protein content of PPARG in subcutaneous adipose tissue, whereas no significant difference for ZNF423 (a transcriptional regulator of preadipocyte determination; Gupta et al., 2010; Huang et al., 2012) was detected in samples between Wagyu and Angus cattle. In addition, quantification of Oil Red-O indicated the trend that more lipids accumulated in Angus SV cells after 14 d of adipogenic inducement. Taken together, these data suggest that Wagyu adipose tissue possessed a trend of lower adipogenesis than that of Angus cattle. As adipocyte differentiation is also the process of growth (mitosis) arrest and subsequent lipid accumulation (Wei et al., 2013), the lower trend of lipid accumulation was consistent with the higher mitogenesis detected in Wagyu SV cells of this study. Another possibility is that the Wagyu SV cells contain more fibrogenic cells, which limits overall adipogenesis.

Interestingly, although Wagyu SV cells possessed higher proliferative ability compared with Angus SV cells, exogenous FGF2 enhanced SV cell proliferation to a greater degree in Angus than in Wagyu cattle. As one of the FGF family members, FGF2 signals through FGFR1 and activates several key signaling cascades, such as MAPK-mediated ERK activation and PI3K (phosphoinositol-3 kinase)/AKT signaling pathway, regulating cell proliferation (Zubilewicz et al., 2001). The phosphorylation of ERK1/2 followed the same trend with the cell proliferation, corroborating that Wagyu SV cells had higher basal proliferative capacity but were less responsive to the stimulation of FGF2.

To identify mechanisms leading to the enhanced proliferation of Wagyu SV cells, we analyzed the expression of an array of autocrine growth factors, including FGF2, EGFR, VEGFA, PDGFRA, HGF, BMP2, TGFB1, TGFB2, TGFB3, FGF7, and FGF10, as well as other related genes including LOX-PP, COL1A1, COL3A1, FN1, and FGFR1. Results indicated that

the relative mRNA expressions of BMP2 and TGFB3 were lower in SV cells of Wagyu compared with those of Angus cattle. Both TGFB3 and BMP2 belong to the TGF-β family, which inhibits cell proliferation but promotes differentiation (Hostettler et al., 2009; Rodrigues et al., 2010). Indeed, TGFB3 negatively regulates cell proliferation via ERK pathway (Lafontaine et al., 2011), which is consistent with our data. Therefore, the differential expression of TGFB3 and BMP2 might partly explain the decreased proliferation of Wagyu compared with Angus SV cells observed in this study. In this study, we used 18S rRNA as the reference gene. Though we have verified the expression of 18S against those of GAPDH and β-catenin as reference genes, the large difference in the abundance of 18S compared to the mRNA expression of target genes might introduce variations during qPCR reaction.

In summary, results from this study show that Wagyu subcutaneous adipose tissue possessed larger adipocytes at 12 mo of age compared with that of Angus cattle. The mRNA and protein expression of PPARG of Wagyu cattle were lower than those of Angus in SV cells and/or adipose tissue, which may play a role in the reduced adipogenesis in Wagyu subcutaneous adipose tissue. Moreover, mitogenesis as well as ERK pathway appeared to be enhanced in SV cells derived from Wagyu subcutaneous adipose tissue. The enhanced mitogenesis as well as ERK pathway are likely due to the lower expression of TGFB3, providing clues and potential targets of further investigations for improving animal production efficiency.

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