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Fat depot-specific differences in pref-1 gene expression and adipocyte cellularity between *Wagyu* and Holstein cattle

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ABSTRACT

Preadipocyte factor-1 (pref-1) is specifically expressed in preadipocytes and acts as a gatekeeper of adipogenesis by maintaining the preadipocyte state and preventing adipocyte differentiation. We hypothesized that the breed differences of adipogenic capacity in cattle could be explained by the expression level of pref-1. In this experiment, we studied the expression level of the pref-1 gene and adipocyte cellularity in subcutaneous and mesenteric adipose tissues of Japanese Black (*Wagyu*) and Holstein fattening cattle. In subcutaneous adipose tissue, there were no significant differences in the pref-1 gene expression levels and adipocyte sizes between the breeds. In contrast, the expression level of the pref-1 gene in mesenteric adipose tissue of Holsteins was significantly higher than that of *Wagyu*. In addition, the size of mesenteric adipocytes in Holsteins was significantly smaller than that of *Wagyu*. These results indicate that the breed differences of fattening cattle affect the expression pattern of the pref-1 gene and adipocyte cellularity in a fat depot-specific manner.

1. Introduction

Adipose tissue growth (adipogenesis) is dependent on cellular events concerning both the differentiation of preadipocytes and the terminal differentiation of adipocytes [1]. Adipogenic transcription factors, the CCAAT/enhancer-binding protein (C/EBP) family (C/EBPα, C/EBPβ, and C/EBPδ) and the peroxisome proliferator-activated receptor γ2 (PPARγ2), play essential roles during adipogenesis [2,3].

Preadipocyte factor-1 (pref-1) is a trans-membrane protein specifically expressed in preadipocytes [4,5]. Pref-1 is a molecular gatekeeper of adipogenesis, which acts by maintaining the preadipocyte state and preventing adipocyte differentiation [6,7]. Therefore, pref-1 expression has been used as a marker of the differentiation stage of adipogenesis and an indicator of preadipocyte density within adipose tissues [8,9].

Beef breeds have been shown to have different capabilities for adipose tissue deposition. Japanese Black (*Wagyu*) cattle are characterized by the ability to accumulate higher amount of adipose tissues than Holsteins [10]. Previously, we indicated that the expression level of the C/EBP family in *Wagyu* adipocytes was significantly higher than in those of Holsteins [11]. A comparative *in vitro* study of bovine preadipocyte differentiation showed that the differentiation potential of preadipocytes in *Wagyu* is higher than in Holsteins [12]. Overexpression of pref-1 inhibits adipocyte differentiation and adipose tissue growth in mice [13]. These results suggest that, in addition to the expression level of adipogenic transcription factors in adipocytes, breed differences of adipogenic capacity could be explained by the expression level of pref-1 in preadipocytes. However, the effects of breed difference on the expression of the pref-1 in bovine adipogenesis are still unclear. The aim of the present study was to elucidate the effects of breed difference on the expression of the pref-1 gene in the adipose tissue of *Wagyu* and Holstein from various anatomical sites (subcutaneous and mesenteric).

2. Materials and methods

2.1. Animals

The experimental design has been described in detail by Yamada et al. [11]. In brief, sixteen fattening steers (*Wagyu* ($n = 8$) and Holstein ($n = 8$), aged 19–24 months) were fed concentrate

Abbreviations: pref-1, preadipocyte factor-1; C/EBP, CCAAT/enhancer-binding protein; PPARγ2, peroxisome proliferator-activated receptor γ2; RPLP0, ribosomal protein large P0.

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(88% total digestible nutrients and 12% crude protein) and orchard grass hay (56% total digestible nutrients and 8% crude protein) ad libitum from 10 months of age until they were slaughtered. Feeds were individually provided in each group. Adipose tissue samples from two types of fat tissues (subcutaneous and mesenteric) were collected at slaughter. The subcutaneous adipose tissue was collected near the 3rd and 4th lumbar vertebrae, and the mesenteric adipose tissue was sampled from the area surrounding the colon. All adipose tissue samples were collected immediately after slaughter. Adipose tissue samples for determining of pref-1 mRNA were stored at −80 °C in the RNA-later reagent (Ambion, CA, USA) until RNA extraction. Thin slices of adipose tissue samples were fixed with osmium tetroxide for determining the adipocyte cellularity as described below. All animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals (Institute of Livestock and Grassland Science).

2.2. RNA isolation and real-time PCR

Pref-1 gene expressions were analyzed by real-time PCR as described previously [14,15]. In brief, total RNA was extracted from adipose tissue using the RiboPure Kit (Ambion) according to the manufacturer's instructions. The first-strand cDNA was reverse-transcribed from 0.5 μg of total RNA using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. Real-time PCR was performed with a Mini Opticon (Bio-Rad, Munich, Germany) using THUNDERBIRD SYBR qPCR Mix (Toyobo) according to the manufacturer's instructions. The primer sequences were as follows: pref-1, 5′-CTC CCA GGC CAT CTG CTT C-3′ (forward) and 5′-ACA TGT GGT TGT AGC GCA GA-3′ (reverse); ribosomal protein large P0 (RPLP0), 5′-CAA CCC TGA AGT GCT TGA CAT-3′ (forward) and 5′-AGG CAG ATG GAT CAG CCA-3′ (reverse). The reaction conditions were designed as follows: initial denaturation at 95 °C for 60 s followed by 40 cycles at 95 °C for 15 s, 55 °C for 15 s, and 70 °C for 30 s. SYBR Green fluorescence was detected at the end of each cycle to monitor the amount of the real-time PCR product formed during that cycle. The specificity of the PCR products was determined by melting curve analysis at the end of each run. The standard curve of each product followed the calculation of respective gene expressions. The expression levels of pref-1 mRNA were normalized by RPLP0 as an internal control [12,14,15].

2.3. Adipocyte cellularity

Adipocyte cellularity was measured as described previously [14,15]. In brief, the samples of adipose tissue were rinsed in 0.154 M of NaCl and then fixed with 50 mM of collidine-HCl buffer (pH 7.4) containing 2% osmium tetroxide. Fixed adipose tissue samples were then placed into 8 M of urea in 0.154 M of NaCl for 48 h at room temperature. Fixed and urea-isolated adipocytes were separated into 0.01% Triton X-100 in a 0.154 M NaCl buffer (pH 10). The adipocyte diameter was measured using WinROOF software (Mitani Corporation, Fukui, Japan). More than 300 adipocytes for each sample were measured.

2.4. Statistical analysis

All results are presented as the means ± S.D. Statistical significance was determined by analysis of variance (ANOVA) followed by Tukey's post hoc test. Values of $P < 0.01$ were considered significant.

3. Results

3.1. Adipocyte cellularity

In subcutaneous adipose tissue, there was no difference in adipocyte cellularity between the breeds (Fig. 1). In contrast, the photomicrographs of osmium-fixed mesenteric adipocytes showed that larger sizes of adipocytes were more abundant in *Wagyu* than in Holsteins (Fig. 2A and B). As compared to the Holstein, the

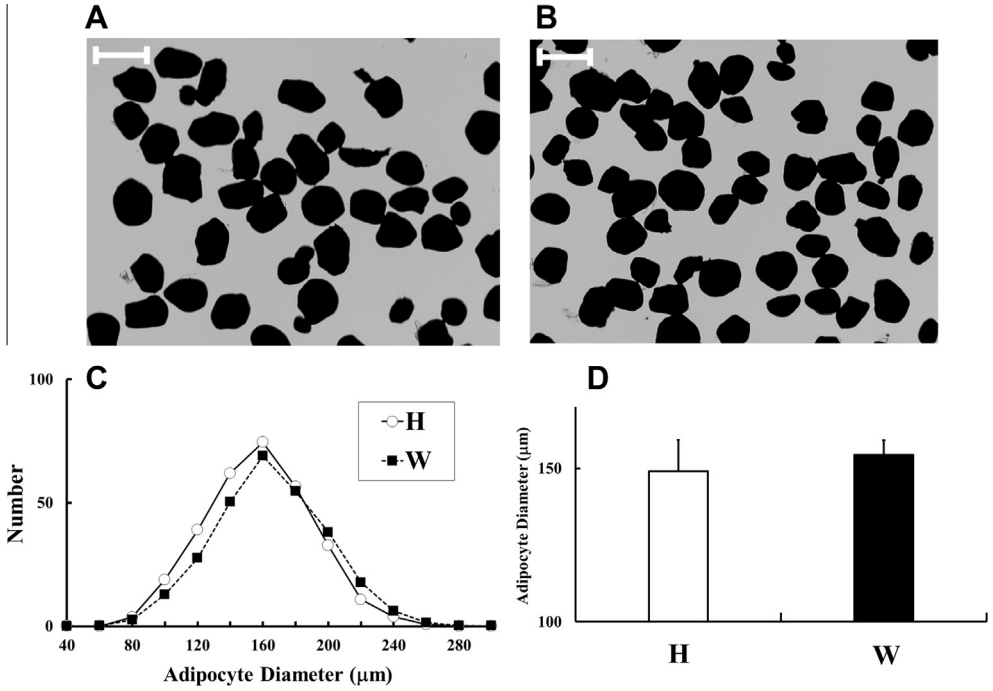

Fig. 1. Adipocyte cellularity in subcutaneous adipose tissue of fattening *Wagyu* and Holstein steers. (A and B) Osmium tetroxide-fixed adipocytes from subcutaneous adipose tissue of (A) Holstein and (B) *Wagyu*. The white scale bar indicates 300 μm. (C) Distributions of the diameters of subcutaneous adipocytes in *Wagyu* (W) and Holstein (H). (D) Mean adipocyte diameter of subcutaneous adipose tissue in *Wagyu* (W) and Holstein (H). The data represent the means ± S.D.

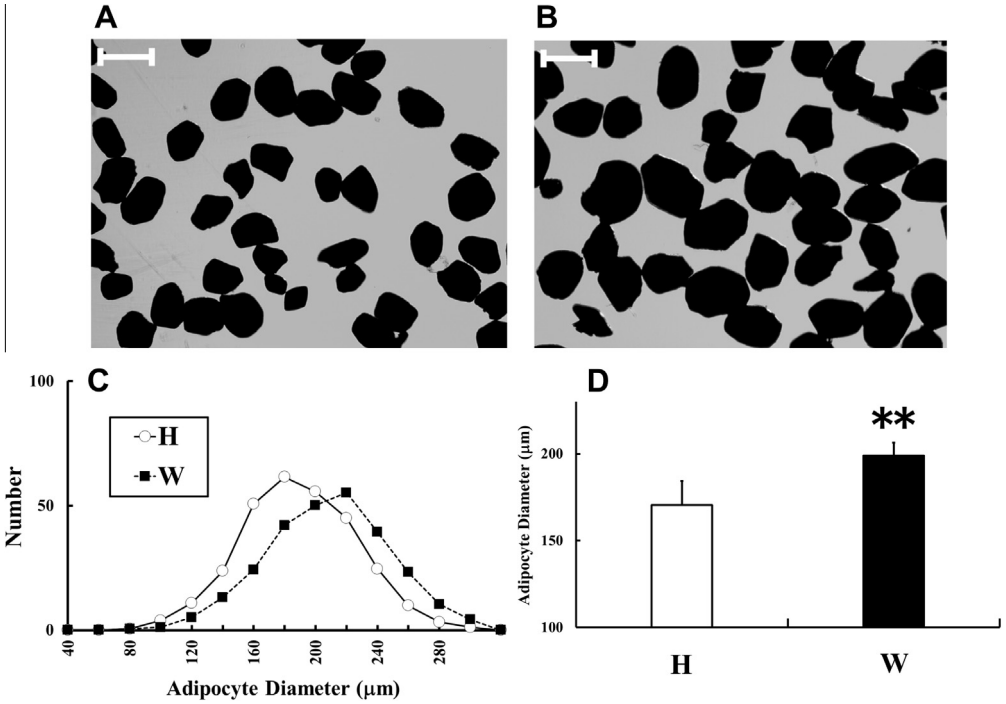

Fig. 2. Adipocyte cellularity in mesenteric adipose tissue of fattening *Wagyu* and Holstein steers. (A and B) Osmium tetroxide-fixed adipocytes from mesenteric adipose tissue of (A) Holstein and (B) *Wagyu*. The white scale bar indicates 300 μm. (C) Distributions of the diameters of mesenteric adipocytes in *Wagyu* (W) and Holstein (H). (D) Mean adipocyte diameter of mesenteric adipose tissue in *Wagyu* (W) and Holstein (H). The data represent the means ± S.D. $^{**}P < 0.01$.

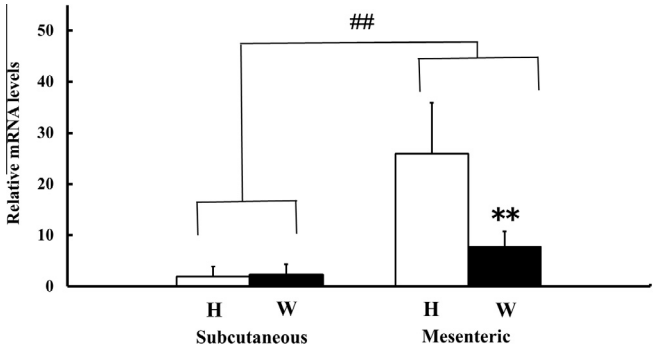

Fig. 3. Expression of Pref-1 mRNA in subcutaneous and mesenteric adipose tissue of fattening *Wagyu* (W) and Holstein (H) steers. RPLP0 mRNA was used as an internal control. The data represent the means ± S.D. **Significant difference between breeds ($P < 0.01$). $^{\#\#}$Significant difference between adipose tissue depots ($P < 0.01$).

mesenteric adipocyte size distribution in *Wagyu* shifted toward larger diameters (Fig. 2C). The mean diameter of mesenteric adipocytes in *Wagyu* was significantly larger than that of Holsteins (Fig. 2D, $P < 0.01$).

3.2. Pref-1 gene expression

Pref-1 mRNA expression levels in bovine adipose tissues are shown in Fig. 3. Mesenteric adipose tissue expressed significantly higher Pref-1 mRNA levels than did subcutaneous adipose tissue ($P < 0.01$). In subcutaneous adipose tissue, there were no significant differences in the pref-1 gene expression levels between the breeds. In contrast, the expression of the pref-1 gene in mesenteric adipose tissue of Holsteins was significantly higher than that of *Wagyu* ($P < 0.01$).

4. Discussion

In the present study, we showed that mesenteric adipose tissues expressed significantly higher pref-1 mRNA levels than did subcutaneous adipose tissues. Tchkonia et al. [16] showed that subcutaneous preadipocytes differentiated better than did visceral preadipocytes. Adams et al. [17] also showed that thiazolidinedione promotes adipogenesis of subcutaneous preadipocytes to a greater extent than preadipocytes derived from the visceral fat depots. Sweter et al. [18] indicated that the expression level of PPARγ2 in visceral preadipocytes is significantly lower than in subcutaneous preadipocytes. In preadipocytes, the expression of adipogenic transcription factors, PPARγ2 and C/EBPα, is suppressed by pref-1 [7,19]. These results suggest that the lower differential ability of visceral preadipocytes might be affected by a higher expression level of pref-1 *via* the downregulation of adipogenic transcription factors.

The present study showed that, although there was no difference in the expression of the pref-1 gene in subcutaneous adipose tissue between the breeds, the expression of pref-1 in the mesenteric adipose tissue of the Holstein was significantly higher than that of the *Wagyu*. We also showed that, although there was no difference in the subcutaneous adipocyte size between breeds, the mean diameter of mesenteric adipocytes in Holsteins was significantly smaller than that of *Wagyu*. Villena et al. [13] reported that the adipocyte size of pref-1-overexpressing mice was significantly smaller than in control mice. Histological analysis of adipose tissue also revealed that adipocytes from pref-1 knockout mice were larger than those from wild-type mice [20]. These results suggest that the smaller size of mesenteric adipocytes in Holsteins might be affected by the higher expression of pref-1. We previously showed that the expression of the C/EBP family (C/EBPβ, C/EBPδ, and C/EBPα) in the mesenteric adipose tissue of Holsteins was significantly lower than that of *Wagyu* [11]. *In vitro* studies using preadipocyte cells indicated that pref-1 downregulates the

expression of C/EBPα [19,21] and suppresses the promoter activity of C/EBPβ and C/EBPδ [22]. These results suggest that lower expression of the C/EBP family in mesenteric adipose tissues of Holstein might be affected by the higher expression of pref-1.

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