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Effect of the anatomical site on telomere length and pref-1 gene expression in bovine adipose tissues

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ABSTRACT

Adipose tissue growth is associated with preadipocyte proliferation and differentiation. Telomere length is a biological marker for cell proliferation. Preadipocyte factor-1 (pref-1) is specifically expressed in preadipocytes and acts as a molecular gatekeeper of adipogenesis. In the present study, we investigated the fat depot-specific differences in telomere length and pref-1 gene expression in various anatomical sites (subcutaneous, intramuscular and visceral) of fattening *Wagyu* cattle. Visceral adipose tissue expressed higher pref-1 mRNA than did subcutaneous and intramuscular adipose tissues. The telomere length in visceral adipose tissue tended to be longer than that of subcutaneous and intramuscular adipose tissues. The telomere length of adipose tissue was not associated with adipocyte size from three anatomical sites. No significant correlation was found between the pref-1 mRNA level and the subcutaneous adipocyte size. In contrast, the pref-1 mRNA level was negatively correlated with the intramuscular and visceral adipocyte size. These results suggest that anatomical sites of adipose tissue affect the telomere length and expression pattern of the pref-1 gene in a fat depot-specific manner.

1. Introduction

Regional differences in adipose tissue metabolism greatly influence the increased risk of metabolic syndrome. Excess visceral adipose tissue accumulation, not the amount of subcutaneous adipose tissue, is causally related to increased risk of metabolic syndrome [1]. Recently, ectopic fat deposition, especially intramuscular adipose tissue accumulation within the skeletal muscle, has been recognized as a new risk factor of metabolic syndrome in humans [2,3]. In beef cattle, the amount of intramuscular adipose tissue is especially important for determining the quality grade of beef. In particular, Japanese black cattle (*Wagyu*) are characterized by the ability to accumulate high amounts of intramuscular adipose tissue [4].

Telomeres are tandem TTAGGG repeats of DNA that cap the ends of chromosomes and promote their stability [5]. Telomere length shortens with every cell division, so telomere attrition is a fundamental aspect of cell proliferation [6]. Moreno-Navarrete et al. [7] reported that the telomere length of subcutaneous adipose tissue in obese subjects was significantly shorter than in non-obese subjects and suggested that telomere length is an adipose tissue expansion marker. Recently, Lakowa et al. [8] showed that the telomere length of subcutaneous adipose tissue in obese subjects was shorter than in visceral adipose tissue. However, the telomere length of the ectopic fat deposition of humans and rodents has not been investigated.

Lakowa et al. [8] also indicated that the telomere length was significantly longer in stromal vascular fraction cells from the visceral adipose tissue than in subcutaneous cells. The stromal vascular fraction of adipose tissues contains non-adipocyte cells within the adipose tissue and is used as an *in vitro* model of preadipocyte differentiation [9,10]. Preadipocyte factor-1 (pref-1) is a transmembrane protein specifically expressed in preadipocytes and acts by maintaining the preadipocyte state as a molecular gatekeeper of adipogenesis [11,12]. Recently, we showed that the breed differences of the bovine adipogenic capacity are affected by the expression level of pref-1 [13]. Adipogenic capacity is closely related to adipocyte size through the expression of pref-1. Overexpression of pref-1 decreased the inguinal adipose tissue weight with a reduction of inguinal adipocyte size in mice [14]. In addition, differences in adipocyte size are related to the parameters of metabolic risk factors in obese subjects [15,16]. However, it remains unclear whether the adipocyte size are related to the telomere

Abbreviations: pref-1, preadipocyte factor-1; RPLP0, ribosomal protein large P0; T/S ratio, telomere to single copy gene ratio.

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length and/or to pref-1 gene expression in a fat depot-specific manner.

In the present study, we investigated fat depot-specific differences in telomere length and pref-1 gene expression in *Wagyu* from various anatomical sites (subcutaneous, intramuscular and visceral).

2. Materials and methods

2.1. Animals

Fifteen fattening Wagyu steers aged 30–31months with a mean body fat percentage of 29.0% (range: 24.0–31.7%) were used in this study. They received a concentrate (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. Adipose tissue samples from three types of fat tissues (subcutaneous, intramuscular and visceral) were collected at slaughter. The left side of the carcass was sectioned between the 3rd and 4th lumbar vertebrae, and the subcutaneous adipose tissue and the intramuscular adipose tissue within the longissimus muscle were collected from this section. Visceral adipose tissue was sampled from the area surrounding the colon. All adipose tissue samples were collected immediately after slaughter. Adipose tissue samples for determining pref-1 mRNA were collected in the RNAlater reagent (Ambion, Foster City, CA, USA) and stored at −80 °C for later RNA extraction. Adipose tissue samples for determining telomere length were snap frozen in liquid nitrogen and kept at −80 °C until analysis. Thin slices of adipose tissue samples were fixed with osmium tetroxide to determine 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. Measurement of pref-1 gene expression

Pref-1 gene expression was analyzed by real-time PCR as described previously [13,17]. In brief, total RNA was extracted from adipose tissue using the RiboPure Kit (Ambion) in accordance with 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 Co., Osaka, Japan) in accordance with the manufacturer's protocol. Real-time PCR was performed with a MiniOpticon system (Bio-Rad, Munich, Germany) using THUNDERBIRD SYBR qPCR Mix (Toyobo) in accordance with 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. The expression levels of pref-1 mRNA were normalized to RPLP0 as an internal control.

2.3. Measurement of telomere length

Telomere length was determined by real-time PCR as described previously [8,18–20]. In brief, genomic DNA was extracted from adipose tissue using the DNeasy Blood & Tissue Kit (Quiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Real-time PCR was performed with a MiniOpticon system (Bio-Rad) using THUNDERBIRD SYBR qPCR Mix (Toyobo) in accordance with the manufacturer's instructions. Two separate PCR runs were performed for each sample, telomere PCR and single copy reference gene (β-globin) PCR. The bovine-specific telomere and β-globin primer sequences were as follows [20]: telomere, 5′-ACA CTA AGG TTT GGG TTT GGG TTT GGG TTT GGG TTA GTG T -3′ (forward) and 5′-TGT TAG GTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA ACA -3′ (reverse); β-globin, 5′-CGG CGG CGG GCG GCG CGG GCT GGG CGG GAA GGC CCA TGG CAA GAA GG -3′ (forward) and 5′-GCC GGC CCG CCG CGC CCG TCC CGC CGC TCA CTC AGC GCA GCA AAG G -3′ (reverse). Telomere sequences were amplified using the following conditions: initial denaturation at 95 °C for 60 s, followed by 30 cycles at 95 °C for 15 s, 55 °C for 15 s, and 70 °C for 30 s. The conditions for β-globin gene amplification were: 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. The telomere to single copy gene ratio (T/S ratio) was calculated as an indicator of telomere length.

2.4. Adipocyte size

Adipocyte size was measured as described previously [13,17]. In brief, the adipose tissue samples were 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), and the adipocyte diameter was measured using WinROOF software (Mitani Corporation, Fukui, Japan). More than 300 adipocytes for each sample were measured.

2.5. 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. The linear regression method was used to

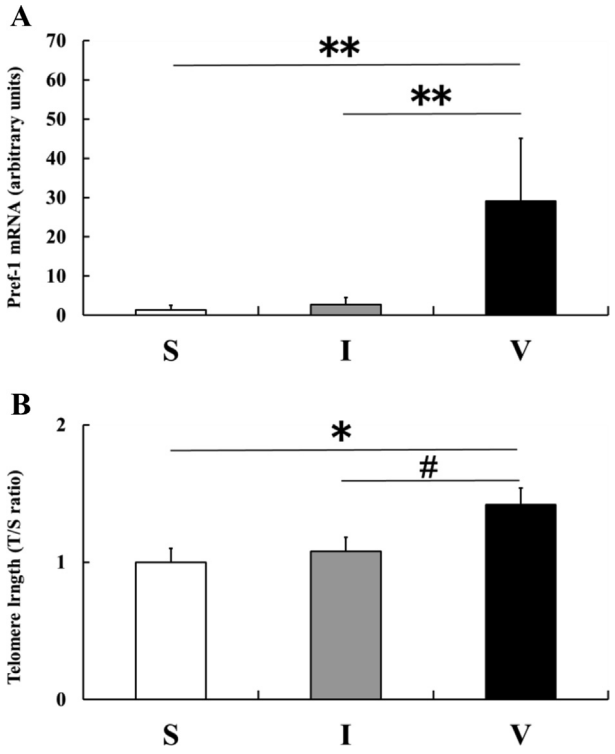

Fig. 1. Fat depot-specific differences in pref-1 mRNA expression and telomere length. A) Expression of pref-1 mRNA in subcutaneous (S), intramuscular (I) and visceral (V) adipose tissue of fattening *Wagyu* steers. The data represent the means ± S.D. **P < 0.01. B) Telomere length differences among subcutaneous (S), intramuscular (I) and visceral (V) adipose tissues of fattening *Wagyu* steers. The data represent the means ± S.D. *P < 0.05, #P = 0.06.

analyze correlations. Values of $P < 0.05$ were considered significant, and $0.05 \leq P < 0.1$ was considered a trend toward significance.

3. Results

3.1. Pref-1 gene expression and telomere length

Visceral adipose tissue expressed higher pref-1 mRNA than did subcutaneous and intramuscular adipose tissues (Fig. 1A). In addition, the telomere length in visceral adipose tissue tended to be longer than that in subcutaneous and intramuscular adipose tissues (Fig. 1B).

3.2. Correlations of adipocyte size with pref-1 gene expression

The telomere length of adipose tissue was not associated with adipocyte size from three anatomical sites (Fig. 2A–C). No significant correlation was found between the pref-1 mRNA level and the subcutaneous adipocyte size (Fig. 2D). In contrast, the pref-1 mRNA expression level was negatively correlated with the intramuscular and visceral adipocyte size (Fig. 2E, F).

4. Discussion

In the present study, we showed that the expression level of pref-1 mRNA in visceral adipose tissue was higher than that of

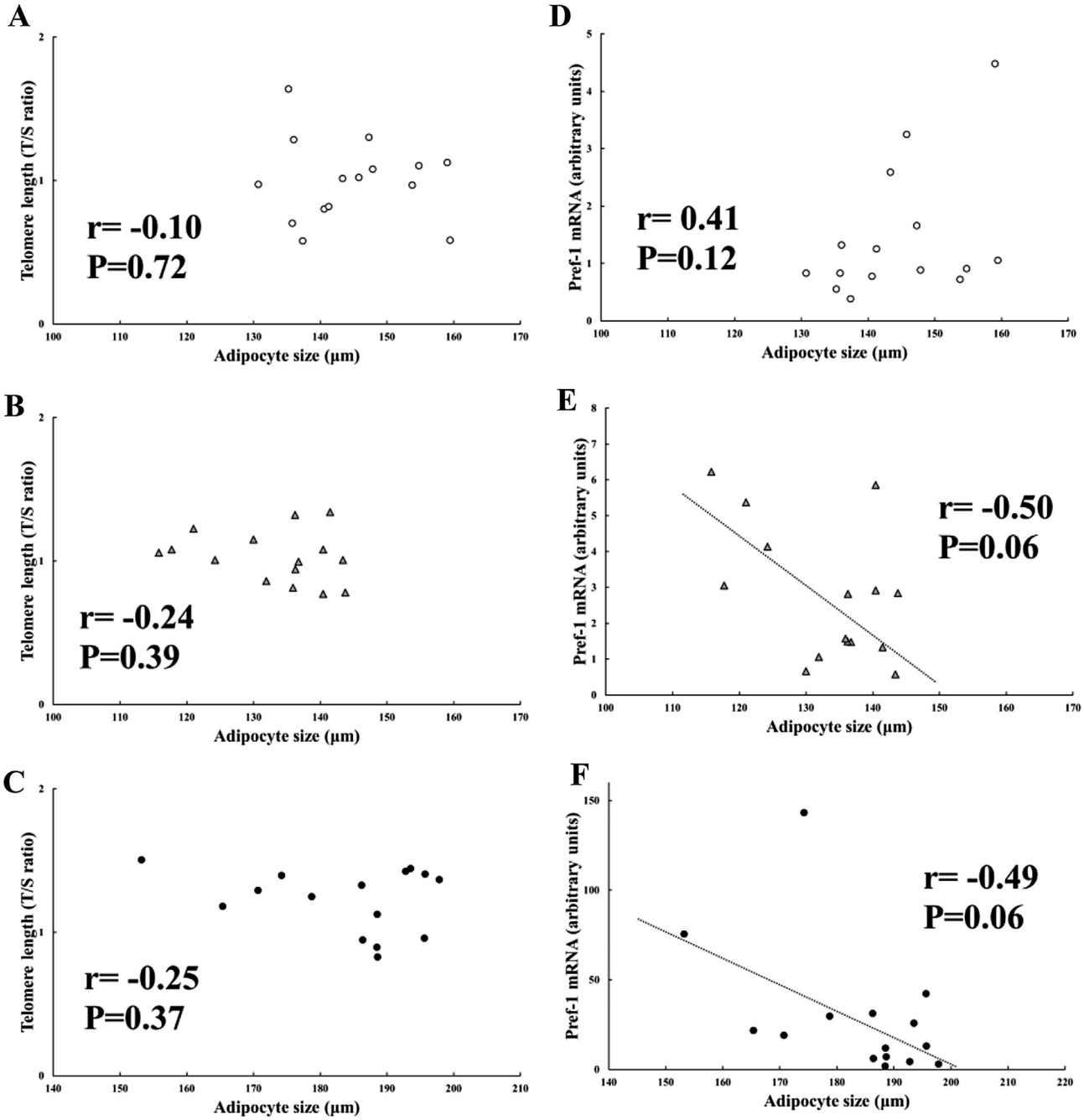

Fig. 2. Correlation of adipocyte size with telomere length and pref-1 mRNA expression. Relationship between telomere length and A) subcutaneous, B) intramuscular and C) visceral adipocyte size. Relationship between pref-1 mRNA expression and D) subcutaneous, E) intramuscular and F) visceral adipocyte size. (○) subcutaneous adipocyte; (▲) intramuscular adipocyte; (●) visceral adipocyte.

subcutaneous and intramuscular adipose tissues. Recent reports showed that the expression of pref-1 in visceral adipose tissue is higher than in subcutaneous adipose tissue [13,21]. Pref-1 inhibits preadipocyte differentiation to adipocyte [11,12]. Tchkonia et al. [22] reported that the differentiation capacity of human visceral preadipocytes is lower than that of subcutaneous preadipocytes. These results suggest that the differentiation capacity of visceral preadipocytes is lower than that of subcutaneous and intramuscular preadipocytes.

We showed that the telomere length in subcutaneous adipose tissue was shorter than that of visceral adipose tissue. Lakowa et al. [8] also reported that the telomere length in obese human subcutaneous adipose tissue was shorter than that in visceral adipose tissue. These results indicate that fat depot-specific differences in the telomere length between subcutaneous and visceral adipose tissues show the same tendency both in obese humans and obese ruminants. We also found that the telomere length in intramuscular adipose tissue tended to be shorter than that in visceral adipose tissue, although there was no difference between subcutaneous and intramuscular adipose tissues. This study is the first to investigate the fat depot-specific differences of telomere length in intramuscular (ectopic) adipose tissue.

A shorter telomere length of adipose tissue reflects an increase in cell proliferation within adipose tissues [7,8]. The present results indicate that the telomere length in subcutaneous and intramuscular adipose tissue is shorter than that of visceral adipose tissue. *In vitro* studies have shown that the proliferation capacity of human subcutaneous preadipocytes is higher than that of visceral preadipocytes [23,24]. These results suggest that subcutaneous and intramuscular preadipocytes are more proliferatively active than visceral preadipocytes.

In the present study, we showed that the telomere length of bovine adipose tissue was not associated with the adipocyte size of three anatomical sites. Bouazzaoui et al. [25] reported that there was no correlation between the telomere length of obese human visceral adipose tissue and visceral adipocyte size. Lakowa et al. [8] indicated that, in humans, there is no difference in telomere length between mature subcutaneous and mature visceral adipocytes. These results suggest that fat depot-specific differences in telomere length may not be affected by mature adipocytes.

We showed that intramuscular and visceral adipocyte size was negatively correlated with pref-1 mRNA expression. Villena et al. [14] reported that the adipocyte size of pref-1-overexpressing mice was significantly smaller than in control mice. Moon et al. [26] also showed that adipocytes from pref-1 knockout mice were larger than those from wild-type mice. These results suggest that intramuscular and visceral adipocyte hypertrophy is affected by the expression level of pref-1. We also showed that there was no correlation between the size of subcutaneous adipocytes and the level of pref-1 mRNA. O'Connell et al. [15] reported that the size of human visceral adipocytes, but not of subcutaneous adipocytes, was strongly related to adiposity and metabolic risk factors. Meena et al. [16] also indicated that the size of human visceral adipocytes was more closely related to the parameters of metabolic risk factors than was the size of subcutaneous adipocytes. In humans, visceral and intramuscular fat accumulations were more strongly associated with adiposity and the risk of metabolic syndrome than was subcutaneous fat growth [1–3]. These results, together with the present findings, suggest that visceral and intramuscular adipocyte hypertrophy may have a greater influence on adipogenesis, especially preadipocyte differentiation capacity, than does subcutaneous adipocyte hypertrophy.

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