Comparison of adipose tissue cellularity in chicken lines divergently selected for fatness

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ABSTRACT After 13 generations of divergent selection for abdominal fatness, 2 chicken lines (a fat line and a lean line) have been established. To clarify the cellular mechanism underlying the differences in fatness between the fat and lean lines, cellularity characteristics of the abdominal adipose tissues were analyzed during the first 7 wk of age by electron microscopy, proliferating cell nuclear antigen staining, and DNA content measurement. The abdominal fat percentage at 7 wk of age in the fat chicken line was 3.8 times that of the lean line, and was accompanied by a 1.3-fold increase in adipocyte diameter and a 2.4-fold increase in adipocyte number. The total cell number of the abdominal fat pad in the fat chicken line was 1.9 times that of the lean line at 7 wk of age. However, no significant difference was observed in the proliferation rate of stroma vascular fraction cells between the 2 chicken lines. These findings suggest that the divergently selected fat and lean chickens have different adipose tissue ontogeny.

Key words: chicken, adipose tissue, morphology, ontogeny

2011 Poultry Science 90:2024–2034
doi:10.3382/ps.2010-00863

INTRODUCTION

Because the obesity problem is becoming worse, adipose tissue biology has been a research hot spot in the life sciences (James, 2004; Ting et al., 2009). Endeavors have been made to investigate the mechanisms underlying adipogenesis in both humans and animals (Hirsch and Batchelor, 1976; Sinnett-Smith and Waddington, 1992; Spalding et al., 2008). It is well accepted that the expansion of adipose tissue involves hyperplastic and hypertrophic growth of adipocytes (Jo et al., 2009; Arner et al., 2010), but the exact mechanisms regulating adipose tissue cellularity are not fully understood, especially in nonmammal vertebrates.

Chickens (*Gallus gallus*) are recognized as an important model organism for fundamental biology (Hedges, 2002) and also an efficient source of lean meat (Cartwright, 1991; Schmidt et al., 2009). Long-term intense selection for increased juvenile growth in broiler chickens has led to increased fat deposition in the chicken abdomen, which is one of the main problems in the broiler industry (Pym, 1987; Emmerson, 1997). Clarification of the morphological characteristics and physiological mechanisms underlying chicken adipose tissue development may enhance efforts to develop successful prevention and treatment strategies to limit the accumulation of excess body fat (Marques et al., 1998).

Two chicken lines have been established in our group by long-term divergent selection on abdominal fat percentage (**AFP**) and plasma very low-density lipoprotein (**VLDL**) concentration (Wang et al., 2007), which provide an ideal bird model to study the mechanisms underlying adipose tissue development and obesity. The objective of this study was to determine the cellularity characteristics of abdominal adipose tissues in 2 divergently selected chicken lines. Our results revealed an ontogenetic pattern change in chicken adipose tissues under sustained selection pressure.

MATERIALS AND METHODS

Birds

The broilers used in this study were derived from the Northeast Agricultural University (**NEAU**) broiler lines divergently selected for abdominal fat content (**NEAUHLF**). The NEAUHLF line has been selected since 1996 by using AFP (abdominal fat weight/BW) and plasma VLDL concentration as selection criteria. The G_0 generation of NEAUHLF came from the same grandsire line originating from the Arbor Acres broiler, which was then divided into 2 lines according to their VLDL concentration at 7 wk of age. From G_1 to G_{13}, birds from each line were raised in 2 hatches, with free access to feed and water. Plasma VLDL concentrations

Received April 26, 2010.
Accepted October 19, 2010.
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were measured for all male birds at 7 wk, and AFP of the male birds in the first hatch was measured after slaughter at 7 wk. Sibling birds from the families with lower (lean line) or higher (fat line) AFP than the average value of the population were selected as candidates for breeding, considering plasma VLDL concentration and the BW of male birds in the second hatch and egg production of female birds in both hatches. The selection procedure and raising conditions have been described in detail previously (Wang et al., 2007). Seventy-five fat male birds and 94 lean male birds of different ages from the 13th-generation population were used in the current study. The fat males used here were the offspring of the families with the highest AFP according to their slaughtered sib information, and the lean males used here were the offspring of the families with the lowest AFP according to their slaughtered sib information. All males were kept in the same environmental conditions and had free access to feed and water. Commercial corn- and soybean-based diets that met all NRC (1994) requirements were provided to the birds. Fat and lean male birds were weighed and killed at d 3, 5, 7, and 10 posthatching, and at 1-wk intervals until 7 wk of age, followed by immediate collection and weighing of the abdominal fat pad. The abdominal fat includes the abdominal fat pad and adipose tissue collected from the walls of the gizzard. Adipose tissues, except for those used in the histological analysis, were immediately placed into liquid nitrogen and stored at −80°C until DNA content measurement and lipid extraction.

Histological Analysis

Fresh abdominal adipose tissues from chickens of different ages were collected and fixed in 4% paraformaldehyde solution. Paraffin-embedded histologic sections were stained with hematoxylin-eosin by using standard procedures at the Histology Laboratory (Department of Histology and Embryology, College of Basic Medical Science, Harbin Medical University).

Transmission Electron Microscopy

Electron microscopy studies were performed at the Electron Microscopy Centre of the Life Science College, Northeast Agricultural University. Briefly, adipose tissue samples were isolated and minced with a razor blade into about 1 mm^3 and fixed with 2% glutaraldehyde and 2% formaldehyde in 0.1 *M* phosphate buffer, postfixed in osmium tetroxide, dehydrated with ethanol, and embedded in Epon (SPI-Pon 812 Epoxy Embedding Kit, SPI supplies, West Chester, PA). Ultrathin sections (50 to 60 nm) were obtained with an RMC Ultramicrotome (RMC Products, Boeckeler Instruments Inc., Tucson, AZ) and stained with uranyl acetate and lead citrate. Specimens were observed with an H-7650 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan). Micrographs were made at original magnifications of 2000 to 20,000 and enlarged photographically to the desired final magnification.

Cellularity Determinations

The adipocyte diameter of 500 to 1,500 cells was determined at each time point for each line using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD) from images of adipose tissue sections obtained by light microscopy (Leica DM LB2, Leica, Heidelberg, Germany; Chen and Farese, 2002). The adipocyte diameter distribution was plotted as a histogram, with each histogram bar representing the average percentage of adipocytes present in a 5-μm-diameter range. Because unilocular adipocytes have 95% lipid content and are spherical in shape, cell volumes can be estimated from their diameter (Trujillo and Scherer, 2006; Sue et al., 2008). The adipocyte numbers of the abdominal fat pad were calculated from the lipid density, mean cell volume, and lipid content of the abdominal fat (DiGirolamo et al., 1998). Total lipid was estimated by chloroform:methanol (2:1, vol/vol) extraction, as described by Folch et al. (1957).

Total DNA Content of Adipose Tissue

The abdominal adipose tissue was dissected and immediately frozen in liquid nitrogen. Genomic DNA was extracted from approximately 50 to 100 mg of tissue using a commercially available acid-phenol reagent (TRIzol, Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The DNA per milligram of tissue was measured by UV spectrophotometry, and the total DNA content was determined by multiplying the obtained DNA concentration by the abdominal adipose tissue weight for each chicken.

Proliferating Cell Nuclear Antigen Immunohistochemistry

Deparaffinized tissue sections (5 μm thick) were rehydrated in serially diluted ethanol, and then immersed in 3% hydrogen peroxide to quench endogenous peroxidase activity. Sections were antigen-retrieved by heating with 10 m*M* citrate buffer (pH 6.0) in a 95°C water bath for 12 min. Nonspecific binding was blocked by incubation with 5% BSA in PBS, followed by incubation with the proliferating cell nuclear antigen (**PCNA**) antibody (PC10, Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:250 dilution overnight at 4°C. Immunostaining was visualized using the streptavidin-biotin peroxidase complex method, with diaminobenzidine-hydrogen peroxide as a chromogen (diaminobenzidine kit, Boster Co. Ltd., Wuhan, China). Sections were counterstained

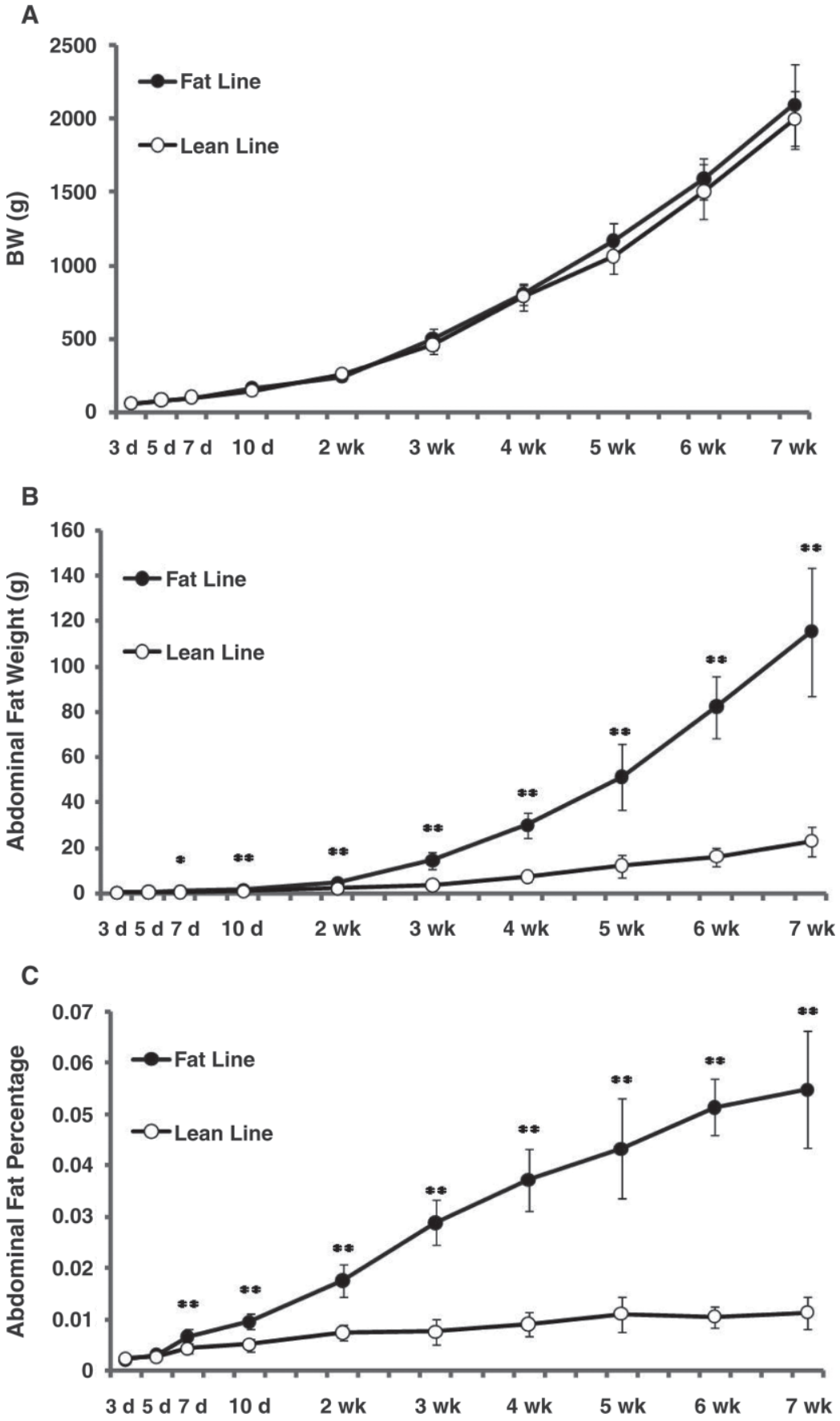

Figure 1. Changes in BW, abdominal fat weight, and abdominal fat percentage of the 13th-generation male of Northeast Agricultural University (NEAU) broiler chickens divergently selected for abdominal fat content during their early growth. A) Body weight changes in divergent lines of lean and fat broilers during the first 7 wk of age. B) Changes in abdominal fat weight during the development of divergent lines of lean and fat broilers. C) Changes in abdominal fat percentage in divergent lines of lean and fat broilers. Mean ± SD, n = 4 to 15. $^{*}P < 0.05$; $^{**}P < 0.01$.

with Mayer's hematoxylin (Sigma Chemical Co., St. Louis, MO) to visualize unlabeled nuclei. The negative control was obtained by omitting the primary antibody on adjacent sections. Chicken liver sections were used as a positive control. To determine the percentage of PCNA-expressing cells, PCNA-staining cells and nonstaining cells in high-powered fields (400× magnification) were counted in different areas of the sections. Approximately 500 to 2,000 cells in different tissue sections per bird specimen were evaluated. The percentage of PCNA-expressing cells for each sample was calculated as the number of PCNA-expressing cells divided by the total number of cells counted multiplied by 100.

Statistical Methods

Results are expressed as mean ± SD. Data were subjected to one-way ANOVA by using JMP 5.1 software (SAS Institute Inc., Cary, NC). Differences were considered significant at $P < 0.05$ unless otherwise specified.

RESULTS

Characteristics of BW and Fat Pad Weight of the 2 Chicken Lines

Fat and lean birds had significantly different fatness traits. The AFP of 7-wk-old male birds was 5.5% in the fat chicken line and 1.1% in the lean line, whereas no significant difference was found in BW between the 2 lines. The difference in abdominal adipose tissue weight between the 2 lines was observed as early as 7 d posthatching (Figure 1). At 7 wk of age, the abdominal fat pad weight of fat birds was approximately 4 times greater than that of lean birds. The characteristics of BW and abdominal fat weight of 7-wk-old chickens are described in Table 1.

Histological Changes in Abdominal Adipose Tissue Between the Fat and Lean Chicken Lines

Morphological studies showed that adipocytes were presented as single adipocytes and clusters of small adipocytes surrounded by stroma cells and vascular structures during the first week posthatching (Figure 2A). Electron microscopy analysis of these small adipocytes revealed that most of them were multilocular adipocytes and were often in close association with capillaries, which may facilitate the exchange of endocrine cytokines or free fatty acids between capillary lumens and adipocytes (Figure 3).

Histological studies and the frequency distribution of adipocyte diameters showed the heterogeneity of the adipocyte size, which ranged from large unilocular to small multilocular adipocytes (Figures 2 and 3). With the development of adipose tissue, unilocular adipocytes became the predominant cell type in the tissue, and the proportion of large adipocytes was higher in the fat chicken line than in the lean line (Figure 2B). At 7 wk of age, the largest proportion of adipocytes (13.7 ± 1.4%) in the lean line was 30 to 35 μm in diameter, whereas the largest proportion of adipocytes (11.6 ± 1.5%) in the fat chicken line was 50 to 55 μm in diameter. Interestingly, as shown in Figure 2B, we also found a second population of adipocytes (10.3 ± 2.9%) with a peak diameter of 25 to 30 μm in the fat chicken line at 7 wk of age, but not in the lean line. The adipocyte diameter distribution pattern of the fat chicken line may involve the enlargement of existing adipocytes and the creation of new, small adipocytes (Sinnett-Smith and Waddington, 1992).

The cellularity characteristics of the abdominal adipose tissues were compared between the 2 chicken lines throughout the first 7 wk posthatching. The significant difference in adipocyte size between the 2 chicken lines appeared at 3 wk of age. In the fat chicken line, before 5 wk of age, hyperplastic growth and hypertrophic growth of adipocytes were involved in the expansion of adipose tissue (Figure 4A and 4B), whereas at 5 to 7 wk of age, the mean cell diameter ceased to increase and tissue expansion was mainly achieved by hyperplastic growth. In the lean chicken line, by contrast, the obvious hypertrophic growth was postponed until 4 wk of age and the fat mass growth mainly depended on the hypertrophic growth from 5 to 7 wk of age. At 7 wk of age, the mean adipocyte diameter of the fat chickens was 1.3 times that of the lean chickens, and the fat cell numbers were 2.4 times those of the lean line (Figure 4A and 4B).

Total Cell Number of Abdominal Adipose Tissue of the Fat and Lean Chicken Lines

Besides mature adipocytes, adipose tissue consists of various cell types, which are collectively called the stroma vascular fraction (**SVF**; Planat-Bénard et al., 2004). The SVF is a heterogeneous cell population in-

Table 1. Characteristics of BW and abdominal fat weight of 7-wk-old chickens[1]

Chicken line	BW (g)	Abdominal fat weight (g)	Abdominal fat percentage (%)
Fat (15)	2,098 ± 276	115 ± 28[A]	5.5 ± 1.1[A]
Lean (10)	1,997 ± 196	23 ± 7[B]	1.1 ± 0.3[B]

[A,B]Means within a row with no common superscript are different ($P < 0.01$).

[1]Values are given as mean ± SD for number of chickens (shown in parentheses).

cluding vascular endothelial cells, fibroblast-like cells, preadipocytes, mesenchymal stem cells, and circulating blood cells. (Gimble et al., 2007). A large portion of SVF cells are considered to have the potential to differentiate into mature adipocytes or to be a supportive structure for adipose tissue development (Hausman et al., 1980; Cinti, 2005). The total cell numbers of the abdominal adipose tissue were evaluated by DNA content measurement. The results showed that the total cell numbers in abdominal adipose tissue in both chicken lines increased steadily from 3 d to 7 wk of age. The significant difference in total cell numbers between the 2 chicken lines was apparent at 10 d posthatching. At 7 wk of age, DNA content of the abdominal fat pad in

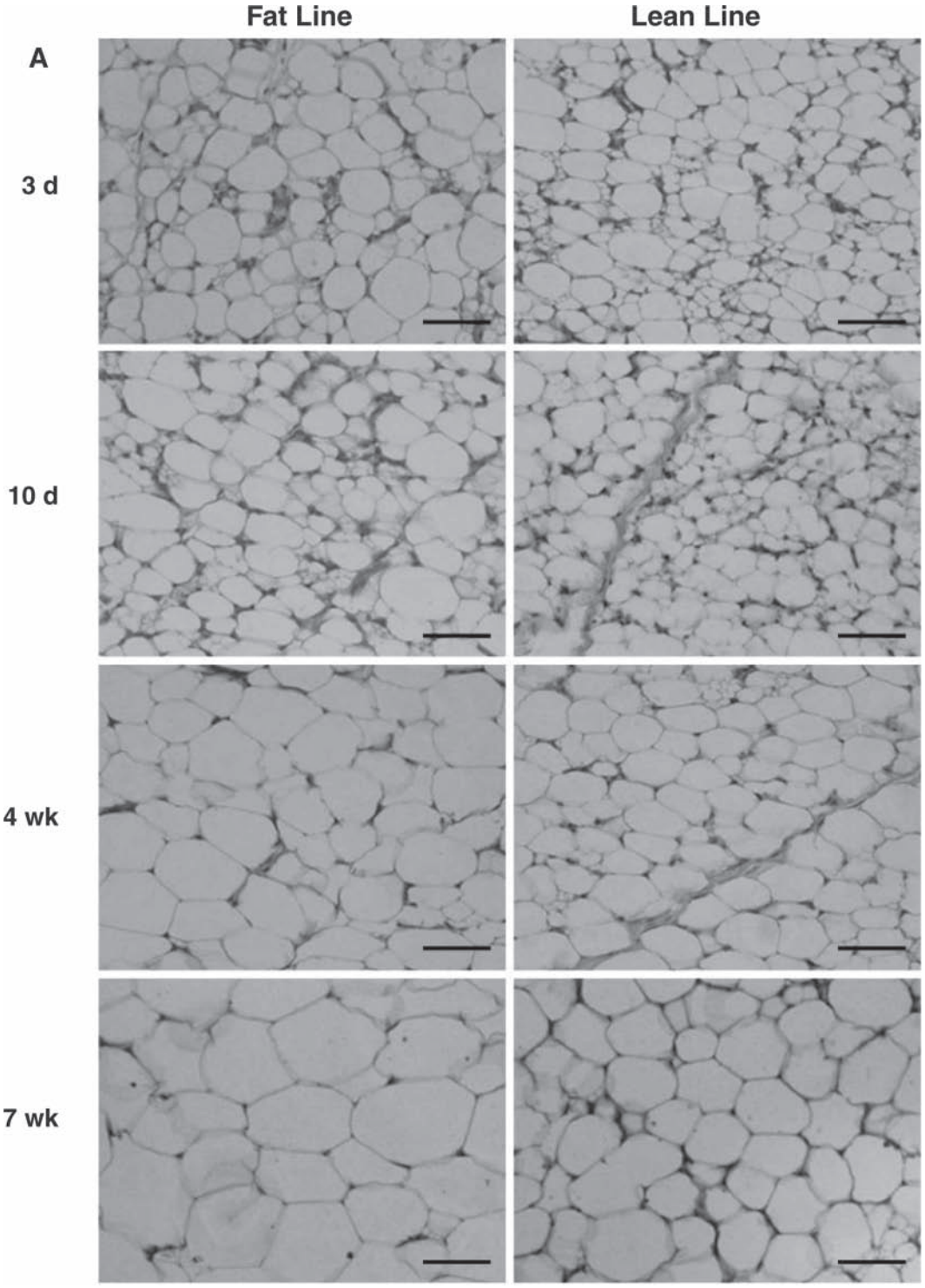

Figure 2. Histological analysis and adipocyte diameter distribution of abdominal adipose tissues of divergent lines of lean and fat broilers. A) Morphological changes in abdominal adipose tissues during the early development of divergent lines of lean and fat broilers. Bar = 50 μm. B) Adipocyte diameter distributions of divergent lines of lean and fat broilers. Results are given as mean ± SD for 3 to 6 independent sections of 3 to 5 chickens for each group. Cells with diameters less than 10 μm were not included.

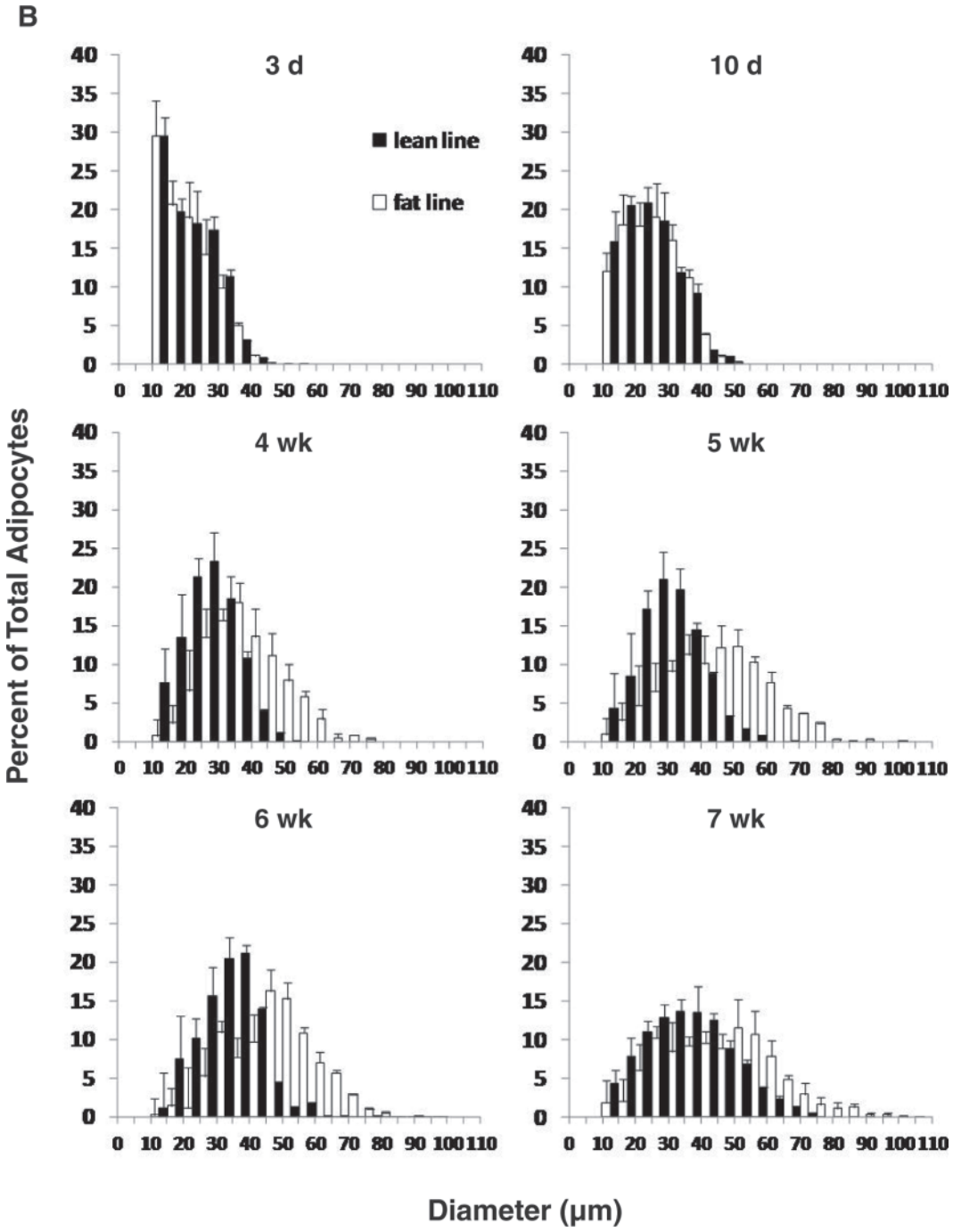

Figure 2 (Continued). Histological analysis and adipocyte diameter distribution of abdominal adipose tissues of divergent lines of lean and fat broilers. A) Morphological changes in abdominal adipose tissues during the early development of divergent lines of lean and fat broilers. Bar = 50 μm. B) Adipocyte diameter distributions of divergent lines of lean and fat broilers. Results are given as mean ± SD for 3 to 6 independent sections of 3 to 5 chickens for each group. Cells with diameters less than 10 μm were not included.

the fat chicken line was approximately 1.9 times that of the lean line (Figure 4C).

Comparison of Cell Proliferation in Abdominal Adipose Tissues Between the Fat and Lean Chicken Lines

To gain insight into the cellular mechanism underlying the differences in fatness between the 2 chicken lines, PCNA immunohistochemistry was performed on abdominal adipose tissues of 10-d-old, 4-wk-old, and 7-wk-old chickens. In both chicken lines, PCNA-positive nuclei were found in the stromal-vascular cells surrounding the mature adipocytes (Figure 5A) and the percentage of PCNA-positive cells declined with age. The percentages of PCNA-positive cells in 10-d-old, 4-wk-old, and 7-wk-old chickens were 33.9, 23.3, and 18.5% in the fat chicken line, and 35.3, 23.5 and 17.9% in the lean chicken line, respectively (Figure 5B). Surprisingly, statistical analyses showed no significant

difference in the percentage of PCNA-positive cells at these 3 time points between the 2 chicken lines, indicating there was no difference in cell proliferation rate of adipose tissue between the 2 chicken lines. Furthermore, we performed a terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling assay (Promega Corp., Madison, WI) in the adipose tissue at 10-d-old, 4-wk-old, and 7-wk-old chickens to compare cell apoptosis between the 2 chicken lines. However, in both chicken lines, cells staining positive by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling were rarely detected at any time point, and no significant difference in apoptosis was observed between the 2 lines (data not shown).

DISCUSSION

In the present study, a longitudinal study on the morphological characteristics of abdominal adipose tissue was conducted in the 2 broiler lines. Histological analysis revealed that the abdominal adipose tissue development of lean birds was decreased in both adipocyte formation and cell enlargement as compared with that of fat birds (Figure 4). These findings were in agreement with previous studies in other chicken populations selected for high or low body fat (Simon and Leclercq, 1982; Hermier et al., 1989), illustrating that selection for the fatness trait results in cellularity changes in adipose tissues.

Previous studies showed that fat cell number and size in broiler-type chickens increased up to 12 to 14 wk of age (March and Hansen, 1977; Hood, 1982; Cartwright, 1991), but any increase in fat deposition thereafter was due to cell hypertrophy (Pfaff and Austic, 1976; Ballam and March, 1979). Our data in the fat chicken line showed that adipocyte hyperplasia occurred from 3 d posthatching to 7 wk of age, and the hypertrophic growth occurred before 5 wk of age (Figure 4A and 4B). During 5 to 7 wk of age, the fat cell size ceased to increase in the fat chicken line, which was not observed in commercial broilers (Hood, 1982). It should be noted that the abdominal adipose tissue weight in the fat chicken line was much heavier as compared with that of other broilers at an equal age or BW (Cartwright et al., 1986; Cartwright, 1991). According to the data from Merkley and Cartwright, the abdominal fat pad weight of our fat birds at 7 wk of age was approximately equal to that of 12-wk-old broilers of the reported population (Merkley and Cartwright, 1989). For this reason, the difference in cell hypertrophy between our fat broilers and other broilers may be explained primarily by the sustained selection pressure on fatness traits, which has led to a high fat storage capacity and the acquisition of a "critical fat cell size" at a younger age (Marques et al., 1998).

In contrast, observations in the lean line showed that the fat cell numbers remained stable between 5 to 7 wk of age. Because the fat pad mass remained unchanged between 8 to 12 wk of age in the lean chicken line (data not shown), it is reasonable to believe that the adipocyte numbers of lean birds would not increase progressively thereafter. These observations may suggest that the ontogeny of adipose tissue in the lean chicken line has been hampered in their juvenile growth period as a consequence of long-term divergent selection for fatness traits. Similar observations were also obtained by Cherry et al. (1984) in a commercial broiler strain, in which the abdominal fat pad growth was primarily due to adipocyte hyperplasia before 4 wk of age, and the accretion of fat at older ages was associated with adipocyte hypertrophy. It should be emphasized that inconsistency exists in some cellularity characteristics of chicken adipose tissues between different laboratories because of a host of factors, such as diet, breed or strain, sex, prior nutritional management, and environmental temperature (Griffin et al., 1987; Pym, 1987; Cartwright, 1991; Donnelly et al., 1993).

It is worth noting that the total cell numbers and fat cell numbers between the 2 chicken lines were almost the same in the first week posthatching. However, because no difference was detected in the SVF proliferation capacity and cell apoptosis (data not shown), it is difficult to explain the disparity in total cell numbers and fat cell numbers between the 2 chicken lines. One possibility is that the difference in fat cell numbers between the 2 lines may account for the differences in undifferentiated cells or the pool of small preadipocytes residing in the fat tissues. During the growing period, these precursors are recruited to become mature adipocytes to a greater extent in fat chickens. The pool of small preadipocytes with a diameter less than 10 μm, which could not be estimated in our studies, may represent such sources. Another possibility is that the newly formed fat cells may arise not only from resident precursor cells, but also from other sources, such as

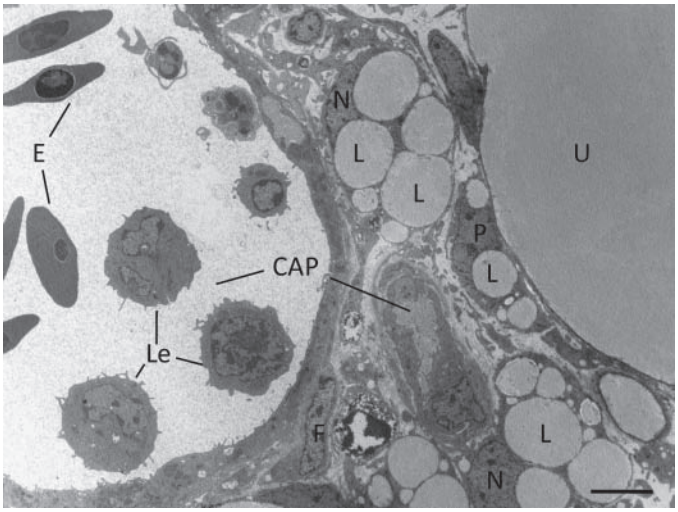

Figure 3. Representative electron micrographs of abdominal adipose tissues of 7-d-old chickens. Small multilocular adipocytes are in proximity to capillaries and unilocular adipocytes. Bar = 5 μm. CAP = capillary lumens; N = nuclei; L = lipid droplets; U = unilocular adipocytes; P = pericytes (probably an earlier stage of preadipocyte development); F = fibroblasts; E = erythrocyte; Le = leukocytes.

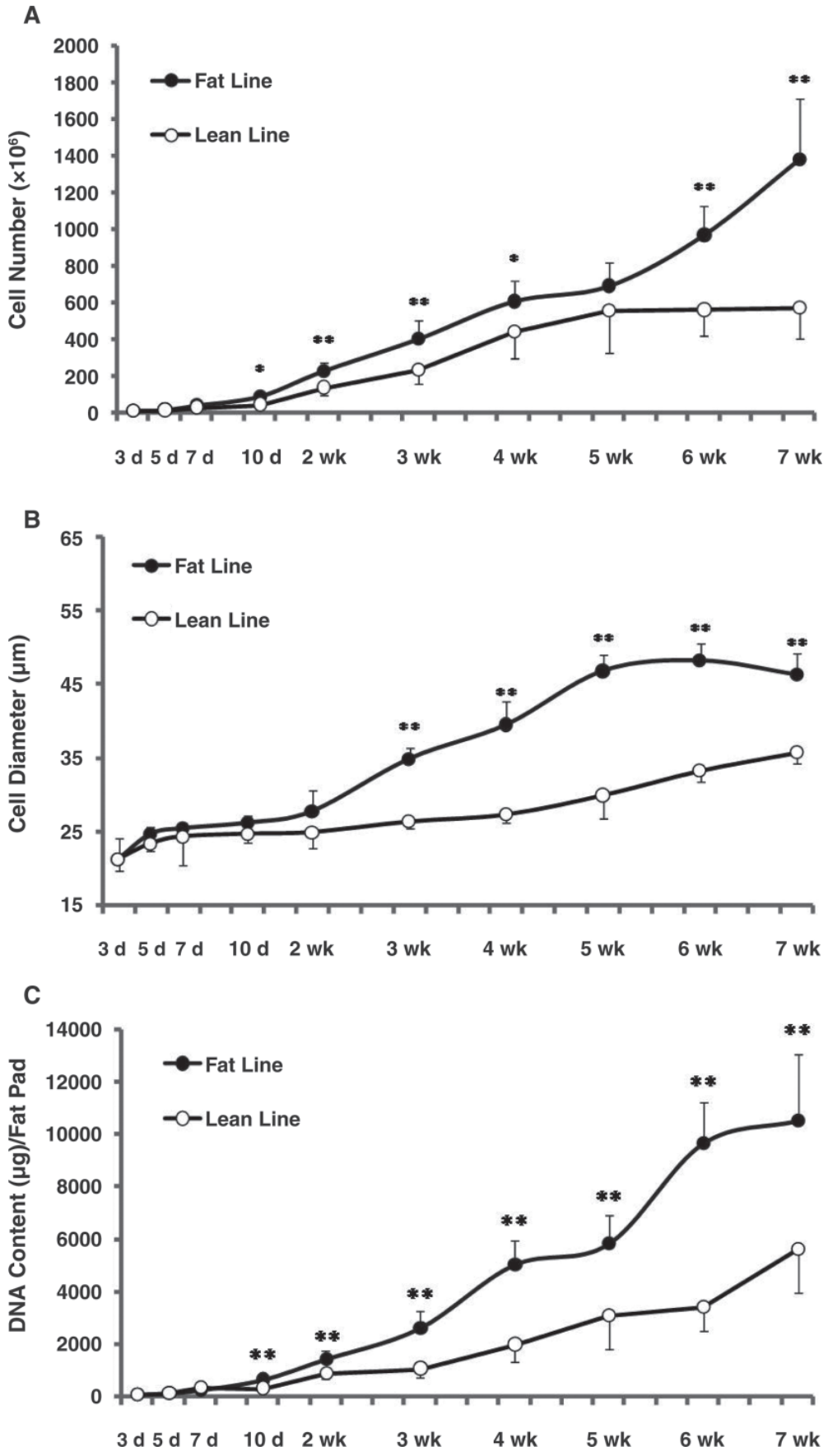

Figure 4. Cellularity characteristics of divergent lines of lean and fat broilers. A) Changes in adipocyte number of chicken abdominal adipose tissue of divergent lines of lean and fat broilers in the first 7 wk of age. B) Changes in adipocyte diameter between the divergent lines of lean and fat broilers. Average adipocyte diameter was determined from 3 to 5 chickens for each group. C) Total DNA content of the abdominal fat pad of divergent lines of lean and fat broilers. Mean ± SD, n = 4 to 15. $^{*}P < 0.05$; $^{**}P < 0.01$.

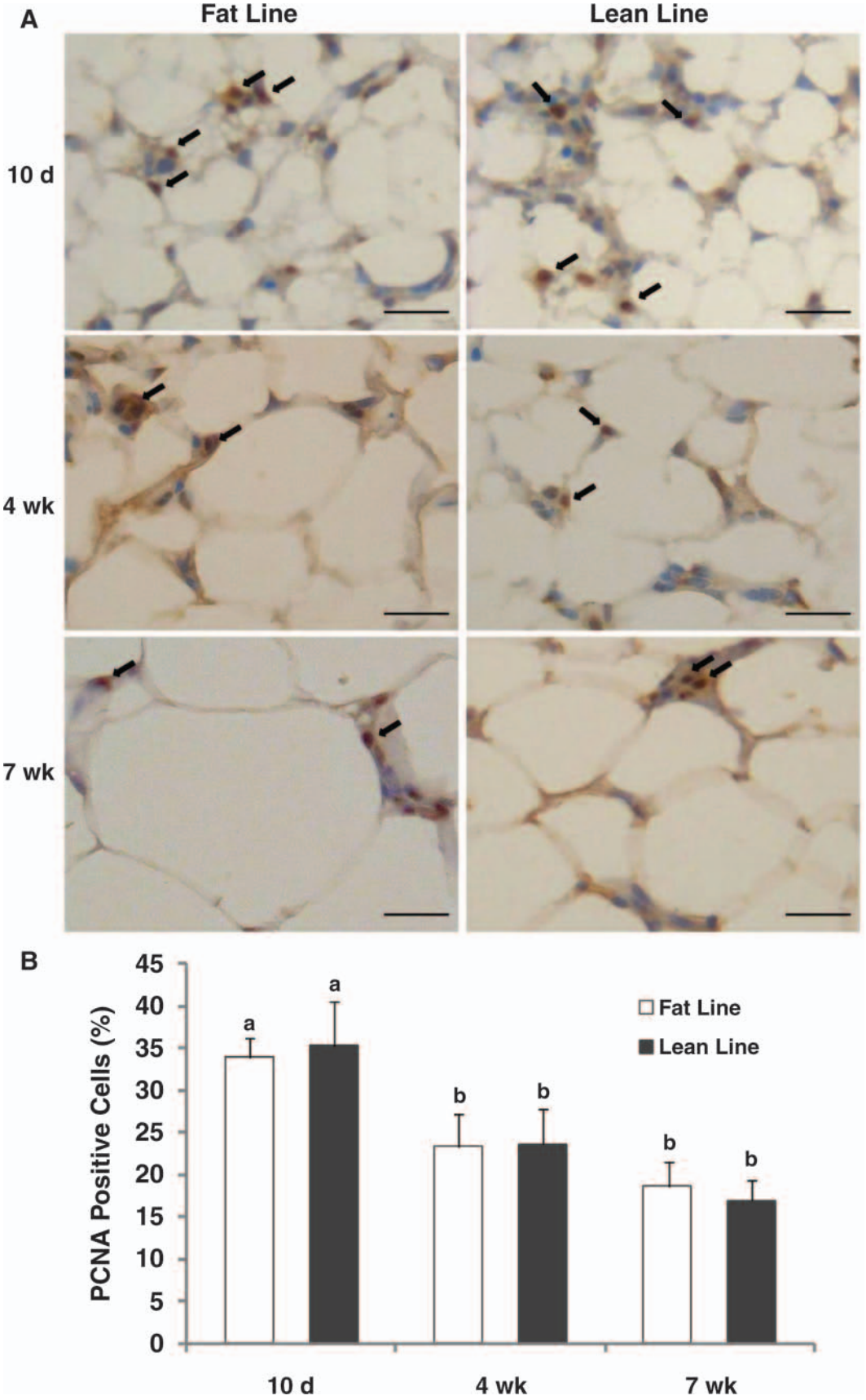

Figure 5. Stroma vascular fraction (SVF) proliferation capacity of abdominal adipose tissues at 10 d, 4 wk, and 7 wk of age. A) Representative pictures of proliferating cell nuclear antigen (PCNA) immunohistochemistry in divergent lines of lean and fat broilers. Arrows indicate PCNA-positive cells. Bars = 20 μm. B) Percentages of PCNA-positive cells in divergent lines of lean and fat broilers at 10 d, 4 wk, and 7 wk of age. Results are given as mean ± SD for 3 independent sections of 3 to 5 chickens for each group. Bars with different letters (a, b) are significantly different at $P < 0.05$. Color version available in the online PDF.

circulating progenitor cells, especially in the fat chickens. This assumption was deduced from some studies in mammals, which suggested that some circulating progenitor cells can serve as a source of adipocyte precursors in adipose tissues (Hong et al., 2005; Crossno et al., 2006). Studies in humans also provided more direct evidence for developmental heterogeneity of adipocytes and preadipocytes within the same adipose depots (Tchkonia et al., 2005, 2006).

Furthermore, although PCNA has been used extensively as a cell proliferation marker, previous studies have shown some inaccuracy in PCNA immunostaining in the assessment of cell proliferation rate (Muskhelishvili et al., 2003). For this reason, we cannot totally exclude the possibility of there being a significant difference in proliferation capacity between the 2 chicken lines. The different proliferation capacities may contribute to the difference in total cell numbers between the 2 chicken lines.

In summary, all these findings suggest that the development of adipose tissue has been promoted in the fat chicken line either in adipocyte enlargement or in fat cell formation, whereas the ontogeny of adipose tissue in the lean chicken line has been hampered in the juvenile growth period as a consequence of long-term divergent selection for fatness traits. Together with the results of SVF proliferation and cellularity characterization, all these data signify a potential relationship between the onset of lipid accumulation and nonresident cell recruitment, which may be a partial explanation for the difference in fatness between the 2 chicken lines. Further research is required to broaden our understanding of the mechanisms underlying the control of adipocyte numbers and the developmental origin of adipocyte progenitor cells.

ACKNOWLEDGMENTS

The authors express their thanks to the members of the Poultry Breeding Group of the College of Animal Science and Technology in Northeast Agricultural University (Harbin, P. R. China) for managing the birds and collecting the data. This work was funded by National 973 Project of China (Ministry of Science and Technology, Beijing, P. R. China; No. 2006CB102105), National 973 Project of China (Ministry of Science and Technology; No. 2009CB941604), and National 863 Project of China (Ministry of Science and Technology; No.2006AA10A120).

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