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Title page

## **Temporal and spatial variations of lipid droplets during adipocyte division and differentiation**

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Abbreviated title: Lipid droplet variations during adipocyte differentiation

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Abbreviations: SVC(s), stromal-vascular cell(s); LD(s), lipid droplet(s); PhC, phase-contrast; DIC, differential interference contrast; ADRP, adipose differentiation-related protein; LD-associated, lipid droplet-associated; PPAR- $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ .

## abstract

By capturing time-lapse images of primary stromal-vascular cells (SVCs) derived from rat mesenteric adipose tissue, we revealed temporal and spatial variations of lipid droplets (LDs) in individual SVCs during adipocyte differentiation. Numerous small LDs (a few micrometers in diameter) appeared in the perinuclear region at an early stage of differentiation; subsequently, several LDs grew to more than 10  $\mu\text{m}$  in diameter and occupied the cytoplasm. We have developed a method for the fluorescence staining of LDs in living adipocytes. Time-lapse observation of the stained cells at higher magnification showed that nascent LDs (several 100 nm in diameter) grew into small LDs while moving from lamellipodia to the perinuclear region. We also found that adipocytes are capable of division and they evenly distribute the LDs between two daughter cells. Immunofluorescence observations of lipid droplet-associated proteins revealed that such cell divisions of SVCs occurred even after LDs were coated with perilipin, suggesting that the “final” cell division during adipocyte differentiation occurs considerably later than that characterized in 3T3-L1 cells. Our time-lapse observations have provided a detailed account of the morphological changes that SVCs undergo during adipocyte division and differentiation.

### **Supplementary key words**

time-lapse observation, fluorescence observation, live cell imaging, visceral adipocyte, adipogenesis, adipose conversion, lipid bodies, lipid droplet-associated proteins.

main text

## INTRODUCTION

The adipose tissue, consisting mainly of adipocytes, functions as an organ for energy storage. However, hypertrophy and hyperplasia of adipocytes in the adipose tissue are associated with obesity, a major risk factor for lifestyle-related diseases. Adipocyte proliferation and differentiation have therefore been investigated in various disciplines such as cell biology, physiology, and pathology. For the past 30 years, 3T3-L1 cells (1, 2) and primary stromal-vascular cells (SVCs), which are derived from inguinal, abdominal, perirenal, retroperitoneal, and epididymal adipose tissues (3-9), were reported to be capable of differentiation into adipocytes. Hence, these cells have been used as in vitro models to clarify cellular and molecular events involved in adipocyte differentiation. According to previous reports, preadipocytes can proliferate until they undergo growth arrest at confluence; subsequently, the growth-arrested cells undergo additional cell division, known as “clonal expansion,” before they start accumulating lipid droplets (LDs) in their cytoplasm (for review, see ref. 10). Furthermore, other reports showed that fully mature adipocytes having large single LDs, referred to as unilocular adipocytes, can also proliferate under specific culture conditions such as “ceiling culture” (11) or “three-dimensional collagen gel culture” (12). However, it has never been clarified whether adipocytes divide during adipocyte differentiation after they start accumulating LDs although they have been considered to lack proliferative activity.

In optical microscopy, LDs appear as well-defined spheres. Accumulation of LDs is widely believed to be the most characteristic function of adipocytes. Observations of LDs using electron microscopy revealed that each LD was surrounded by a phospholipid monolayer (13), and this

suggested that LDs were initially produced from the endoplasmic reticulum in undifferentiated cells (14). It was also revealed that vimentin intermediate filaments form cage-like structures around LDs (15), and that the disruption of these structures inhibited the formation of LDs (16). In molecular biology and biochemistry, various proteins such as adipose differentiation-related protein (ADRP, also known as adipophilin), perilipin, and caveolin, have been found in (or on the surface of) the phospholipid monolayer of LDs (17-20; for review, see refs. 21, 22). It was reported that the transition of lipid droplet-associated (LD-associated) proteins from ADRP to perilipin in 3T3-L1 cells occurred 3 days after the induction of adipocyte differentiation (17). Furthermore, these proteins have been reported to play critical roles in the formation and subsequent enlargement of LDs (23-26).

As mentioned above, not only LD structures but also the molecular events underlying the LD dynamics at specific stages of adipocyte differentiation has recently become clear. In contrast, temporal and spatial variations of the LDs in individual cells throughout the entire course of adipocyte differentiation have never been actually observed. For example, electron microscopy and immunofluorescence microscopy require the fixation of cells, and hence temporal variations of LDs in identical cells cannot be observed. On the other hand, conventional optical microscopy facilitates the observation of identical cells throughout the course of adipocyte differentiation because the fixation of cells is not required. The time-lapse observation would clarify the location of LD formation and mechanisms involved in the subsequent enlargement of identical LDs. Moreover, the observation would determine whether adipocytes divide during adipocyte differentiation.

In the present study, we captured the time-lapse images of SVCs derived from the rat mesenteric adipose tissue throughout the entire course of adipocyte differentiation. The time-lapse observations demonstrated temporal and spatial variations of LDs in individual cells. Numerous

small LDs (a few  $\mu\text{m}$  in diameter) appeared in the perinuclear region at an early stage of differentiation, after which several LDs enlarged. Finally, the cytoplasm was completely filled with several large LDs (more than 10  $\mu\text{m}$  in diameter). Furthermore, we developed a method for the fluorescence staining of LDs in living adipocytes, and we observed the temporal and spatial variations of LDs at a higher magnification. The results revealed that nascent LDs (several 100 nm in diameter) developed in the lamellipodia, and they subsequently grew into small LDs while moving toward the perinuclear region. Our time-lapse observations also showed that contact inhibition was not required for the induction of adipocyte differentiation from the SVCs, and adipocytes were capable of division. In such cell division, adipocytes evenly distributed LDs between two daughter cells. Immunofluorescence observations of LD-associated proteins revealed that such cell division of adipocytes occurred even after the LDs were coated with perilipin. These findings suggest that the “final” cell division during adipocyte differentiation from primary SVCs occurs considerably later than that characterized in 3T3-L1 cells.

## MATERIALS AND METHODS

### **Cells and cell culture**

We used a commercially available primary culture system (Visceral Adipocyte Culture Kit-1; Primary Cell Co., Ltd., Sapporo, Japan), a kind gift from the manufacturer. The culture system, comprising SVCs derived from the mesenteric adipose tissue of Sprague-Dawley rats and a culture medium based on DMEM/F12, was previously established by Shimizu et al. (27). In the previous study (27), the culture medium was supplemented with 10% newborn calf serum, 17  $\mu\text{M}$  pantothenic acid, 33  $\mu\text{M}$  biotin, 100  $\mu\text{M}$  ascorbic acid, 1  $\mu\text{M}$  octanoic acid, 50 nM triiodothyronine, 2.5 mM

nicotinamide, 10 µg/ml insulin, 100 units/ml penicillin, and 100 µg/ml streptomycin as optimal conditions for the induction of adipocyte differentiation from the SVCs. When the SVCs were cultured in the medium for 9 days, more than 80% of the cells expressed the adipocyte phenotype with an accumulation of many LDs in their cytoplasm. We used the culture medium continually throughout the course of adipocyte differentiation in the following experiments.

For cell culture, cryopreserved SVCs were rapidly thawed in a 37°C water bath. To remove the cryopreservation medium from the cell suspension, we carried out the following procedure twice: the supernatant was replaced with the culture medium after centrifugation at 1000 rpm for 3 min, and then the settled cells were resuspended. The obtained cell suspension was placed at an approximate concentration of  $1 \times 10^5$  cells/dish into glass petri dishes, and a suitable amount of fresh medium was added to each dish. The dishes in which cells were inoculated were maintained at 37°C and 5% CO<sub>2</sub> in a humidified incubator during the cell culture period. Following an overnight incubation, the medium was replaced with fresh culture medium to remove floating cells; thereafter, the medium was replaced once every 2 days.

### **Time-lapse observation**

The sealing of culture petri dishes was reported to be an effective method for 5-day time-lapse observations of epithelial cells (28). For the time-lapse observations of SVCs during adipocyte differentiation (several days), we prepared sealed dishes according to the following procedures. Fresh culture medium was adjusted to 37°C and pH 7.0, and a lipid emulsion (Intrafat; Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) containing 1.2 g/100 ml lecithin, 2.25 g/100 ml glycerol, and 20 g/100 ml soybean oil was added at 0.1% to the medium. The addition of lipid emulsion was reported to promote the enlargement of LDs during adipocyte differentiation (27), although the lipid

emulsion was not essential for adipocyte differentiation. The dish was completely filled with the culture medium with the lipid emulsion, and was then sealed with a sterile coverslip and silicone grease (Dow Corning Toray Co., Ltd., Tokyo, Japan). Since it was impossible to exchange the medium once the dish was sealed, the increased metabolic activities of adipocytes might have resulted in the induction of a decrease in pH due to the release of free fatty acid. In the present experiments, we confirmed that the apparent pH of the medium indicated by phenol red did not change significantly in a sealed petri dish. This indicated that the cells were supplied with a sufficient amount of medium.

Time-lapse observations were performed using a phase-contrast (PhC) microscope (IX71; Olympus, Tokyo, Japan) or a differential interference contrast (DIC) microscope (IX81; Olympus). Each microscope was equipped with a 20× objective lens and a digital charge-coupled device camera (DP70; Olympus). The upper part of the microscope—including the sample stage, the objective lens, and the CCD camera—was enclosed in an acrylic resin box in which the temperature was maintained at 37°C. A sealed dish was placed on the sample stage, following which time-lapse images were captured every 5 min. The series of captured images was converted into a movie using MediaStudio<sup>®</sup> Pro software (Ulead Systems, Inc., Yokohama, Japan).

### **Fluorescence observations of LDs in living cells**

BODIPY 493/503 (Molecular Probes, Eugene, OR) and Hoechst 33342 (a kind gift from Dr. K. Kabayama, Hokkaido University, Sapporo, Japan) were used for the fluorescence staining of LDs and nuclei in living cells. BODIPY 493/503, a specific probe for intracellular LDs (29), was dissolved in DMSO to produce 5 mM stock solution similar to the method used in a previous report (30). Hoechst 33342 was dissolved in sterilized distilled-deionized water to produce 2 mg/ml stock

solution. Both stock solutions were added directly to the culture medium at respective final concentrations of 0.5  $\mu$ M (BODIPY 493/503) and 2  $\mu$ g/ml (Hoechst 33342). The cells were stained in a petri dish for the last 10 min of the cell culture period. The stained cells were washed twice with fresh culture medium. The dish was then sealed as described above, except that the lipid emulsion was not added to the medium.

The DIC microscope that we used for time-lapse observations was equipped with an additional 60 $\times$  oil immersion objective lens and epifluorescence optics for fluorescence observations. The microscope system was controlled by MetaMorph<sup>®</sup> software (Nihon Molecular Devices, Tokyo, Japan). Fluorescence images of BODIPY 493/503 and Hoechst 33342 were independently acquired by switching to the appropriate excitation/emission filter sets. A DIC image corresponding to the fluorescence images was also acquired. These three types of images were taken once every 5 min. Two fluorescence images of BODIPY 493/503 and Hoechst 33342 were merged to obtain a single image, and the series of merged images overlaid on corresponding DIC images was converted into a movie, as described above.

### **Immunofluorescence observation**

SVCs were inoculated in petri dishes and were cultured in the abovementioned culture medium for 2 days after inoculation; thereafter, they were cultured in the culture medium supplemented with 0.1% lipid emulsion. At various time points after the inoculation, the cells were washed twice with PBS and fixed with 4% formaldehyde in PBS for 10 min, and again they were washed twice with PBS. The fixed cells were permeabilized with 0.01% digitonin (Sigma-Aldrich, St. Louis, MO) in PBS for 10 min and washed twice with PBS, and were then incubated with PBS containing 0.5% BSA for 30 min. The permeabilized cells were incubated with primary antibodies in PBS containing

0.5% BSA for 1 h and were rinsed with PBS containing 0.5% BSA three times. The cells were then incubated with secondary antibodies in PBS containing 0.5% BSA for 1 h and were rinsed three additional times. ADRP was detected by using 5  $\mu\text{g/ml}$  anti-ADRP (PROGEN Biotechnik, Heidelberg, Germany) as the primary antibody and 0.4% Alexa Fluor 488-labeled anti-mouse IgG (Molecular Probes) as the secondary antibody. Perilipin was detected with 5  $\mu\text{g/ml}$  anti-perilipin (Sigma-Aldrich) as the primary antibody and 0.4% Alexa Fluor 546-labeled anti-rabbit IgG (Molecular Probes) as the secondary antibody. To observe the cell nuclei along with ADRP and perilipin in some experiments, cells were incubated with 2  $\mu\text{g/ml}$  Hoechst 33342 for 10 min after the incubation with secondary antibody. Fluorescence images and corresponding DIC images were taken using the DIC microscope that was used for fluorescence observations of LDs.

## RESULTS

### **Time-lapse observation of adipocyte differentiation**

To observe the morphological changes that SVCs undergo during adipocyte differentiation, we used PhC microscopy to capture time-lapse images of SVCs cultured in the sealed petri dishes. The observation was initiated 3 days after the cells were inoculated in the dish, and it continued for 5 days. Typical time-lapse images of SVCs are shown in Fig. 1. The actual images taken at every 5 min were converted into a movie (see Movie 1 in the supplemental data). At 0 h, the fibroblast-like cells adhered and spread on the dish, and approximately 40% of the dish area was covered with the cells. Although a confluent monolayer did not form, some cells had already expressed the adipocyte phenotype, as evidenced by the accumulation of LDs (arrows in Fig. 1). At 1 day after the inoculation, most cells had a long and slender shape, and no cells accumulated LDs (data not shown).

Based on these results, it can be considered that the cells with LDs are differentiated “adipocytes” from the SVCs inoculated in the dish. From 0 through 100 h, cells proliferated by cell division and differentiated into adipocytes. The LDs that accumulated in each adipocyte grew from a few micrometers to more than 10  $\mu\text{m}$  in diameter during this period. These observations are consistent with a previous report (27). Differentiated adipocytes as well as undifferentiated cells showed movement on the dish. The adipocytes tended to spontaneously aggregate with each other as if to form a colony or a spheroid. In the latter half of the observation period, an aggregation of adipocytes was clearly observed (arrowhead in Fig. 1). At 100 h, >80% of the dish area was covered with adipocytes and other cells.

#### **Enlargement of LDs in individual cells**

To investigate the enlargement of LDs in greater detail, we observed spatial and temporal variations of the LDs in individual cells throughout the time course of adipocyte differentiation. The observation performed using DIC microscopy facilitated the identification of boundaries between neighboring LDs more clearly than that using PhC microscopy. Figure 2 shows typical temporal and spatial variations of the LDs in individual cells (see also Movie 2 in the supplemental data). Small LDs, which were approximately a few micrometers in diameter, appeared in the perinuclear region at 0 h, corresponding to 3 days after inoculation. For the following 8 h, the number of LDs increased gradually, whereas the size of each LD showed only slight variation. At 8 h, the entire cell body, except the nucleus, was completely filled with many LDs. From 8 to 24 h, a change in the cells was observed—from containing smaller and numerous LDs to containing larger and fewer ones. In this period, the nucleus moved to the cell periphery, apparently to accommodate the large LDs that occupied the center of the cell body. Furthermore, a morphological change in cell shape, as the cells

converted from a fibroblast-like shape (lateral length of over 80  $\mu\text{m}$ ) to a spherical shape (lateral length of 40  $\mu\text{m}$ ), was accompanied by the enlargement of LDs. Several LDs (9  $\mu\text{m}$  in diameter) occupied the central region of the cell, and smaller ones were distributed outside this region at 24 h. Thereafter, the large LDs continued to grow with time. From 28 to 36 h, the increase and the subsequent decrease in the number of small LDs were observed again in the peripheral region (see Movie 2).

Further, when we analyzed temporal and spatial variations of the LDs in other cells, the time at which small LDs initially appeared in the perinuclear region (corresponding to 0 h in Fig. 2) was found to be  $76.5 \pm 13.4$  h (mean  $\pm$  SD,  $n = 44$  cells in three experiments) after inoculation. The duration of the LD enlargement process (corresponding to 0–40 h in Fig. 2) in individual cells was found to be  $40.9 \pm 12.5$  h (mean  $\pm$  SD,  $n = 23$  cells in three experiments).

### **Emergence of nascent LDs and subsequent formation of small LDs**

To clarify where initial LDs form, we performed time-lapse observations of adipocytes at higher magnification (submicron scale). In this case, since cells usually contain many types of vesicles, it is also necessary to distinguish LDs from other vesicles. To satisfy these requirements, we attempted fluorescence staining of LDs in living adipocytes. Figures 3A and 3B show a fluorescence image and a corresponding DIC image, respectively. Numerous spherical spots (less than 1  $\mu\text{m}$  in diameter) were observed in the fluorescence image (Fig. 3A), and these spots were consistent with the well-defined spherical structures observed in the DIC image (Fig. 3B). This result verifies that LDs can be selectively stained in living adipocytes. The “nascent” LDs were distributed diffusely in the cell body (arrowhead in Fig. 3A) or aggregated in the perinuclear region (arrow in Fig. 3A). Cells containing no obvious LDs were regarded as undifferentiated cells or non-adipocytes.

Temporal and spatial variations of nascent LDs in a differentiated adipocyte are shown in Fig. 4 (also see Movie 3 in the supplemental data). Nascent LDs were developed continuously throughout the lamellipodia during the observation period. The developing LDs were not stationary but moved linearly or unsteadily backward toward the lamellipodium/cell body transition zone. While moving, some of the LDs increased in size (i.e., developed into small LDs). Once they reached transition zone, the small LDs became stationary.

### **Cell division during adipocyte differentiation**

We observed that adipocytes are capable of division even during the process of differentiation. Figure 5 shows typical time-lapse images of the cell division (also see Movie 4 in the supplemental data). Time 0 min corresponds to 5 days after inoculation. At 30 min, an adipocyte, indicated by an arrow in Fig. 5, accumulated 138 LDs (up to a few  $\mu\text{m}$  in diameter) in the cell body. From 30 to 60 min, the adipocyte suddenly acquired a spherical shape. The spherical adipocyte divided into two daughter cells within the next 30 min. LDs were also observed in the daughter cells immediately after the cell division. After 90 min, the individual cells extended again on the dish, and then migrated in different directions. At 120 min, 80 LDs were contained in one daughter cell and 63 LDs were contained in the other. When we counted the number of LDs in the six observed cases of cell division, the ratio of the total number of LDs in the two daughter cells to that in the corresponding parent cell was found to be  $101.7\% \pm 11.7\%$  (mean  $\pm$  SD,  $n = 6$ ). Similarly, the ratio of the number of LDs in one daughter cell to that in the corresponding parent cell was found to be  $50.0\% \pm 12.1\%$  (mean  $\pm$  SD,  $n = 12$  daughter cells). These quantitative data suggest that LDs in a parent adipocyte are evenly distributed between two daughter cells without fusing or dividing.

In three experiments, only 12 of the 48 adipocytes that started accumulating LDs were observed

to divide into two daughter cells. Further, it was observed that the time at which these 12 adipocytes underwent cell division ranged from 58 to 118 h after inoculation. As was expected, undifferentiated cells were also observed to undergo cell division. The adipocyte shown in Fig. 5 also divided in a similar manner 17 h before the images were taken (data not shown). Thus, adipocytes were capable of division at least twice after initiating the accumulation of LDs.

### **Transition of LD-associated proteins during adipocyte differentiation**

To investigate the transition of LD-associated proteins during adipocyte differentiation from SVCs, we performed immunofluorescence staining of ADRP and perilipin at various time points after SVCs were inoculated (Fig. 6A). At 36 h after the inoculation, ADRP showed a punctate pattern, whereas perilipin was not detectable. The numerous dots of ADRP aggregated in perinuclear regions of fibroblast-like cells, and were consistent with nascent LDs in the corresponding DIC image. Perilipin as well as ADRP was expressed in SVCs at 62 h. At this stage, LDs containing ADRP, perilipin, or both (green, red, or yellow arrowheads in Fig. 6A) were observed equally. LDs containing only perilipin tended to be larger and better defined than those containing only ADRP or both ADRP and perilipin. At 87 h, LDs grew up to 4  $\mu\text{m}$  in diameter. Perilipin was strongly localized on the surface of almost all LDs, appearing as rings surrounding them. In contrast, the presence of ADRP was negligible, and it showed diffused distribution in the cytoplasm rather than exclusively on the surface of LDs. The temporal and spatial variations of ADRP and perilipin in SVCs during adipocyte differentiation are consistent with those in 3T3-L1 cells reported previously (17).

To determine the timing of perilipin expression and that of transition of LD-associated proteins in greater detail, we also performed immunofluorescence staining of ADRP and perilipin at 16, 72, and 135 h after the inoculation (data not shown) as well as at the time points shown in Fig. 6A.

When the number of perilipin-expressing cells was counted in 20 cells from 5 to 7 immunofluorescence images at each time point, it was found that 0, 1, 13, 16, 16, and 18 of 20 cells expressed perilipin at 16, 36, 62, 72, 87, and 135 h, respectively. When the expression and localization of ADRP were also assessed in the perilipin-expressing cells, ADRP was co-localized with perilipin on the surface of LDs in 9 of 13 and 8 of 16 perilipin-expressing cells at 67 and 72 h, respectively. In contrast, the localization of ADRP on LDs was not clearly observed in 15 of 16 perilipin-expressing cells at 87 h; thus, the LDs were coated with only perilipin. The quantitative data revealed that 80% of the SVCs expressed perilipin by 72 h after the inoculation, and >90% of the perilipin-expressing SVCs almost completed the transition of LD-associated proteins from ADRP to perilipin by 87 h after the inoculation.

#### **Relationship between composition of LD-associated proteins and LD size or cell division**

We examined the relationship between LD size, represented by the diameter of the largest LD contained in each cell, and composition of the LD-associated protein at 72 h. The sizes of LDs coated with only ADRP and with both ADRP and perilipin were found to be  $0.80 \pm 0.19 \mu\text{m}$  (mean  $\pm$  SD,  $n = 4$ ) and  $1.80 \pm 0.55 \mu\text{m}$  (mean  $\pm$  SD,  $n = 8$ ) in diameter, respectively. Similarly, the size of LDs coated with only perilipin was found to be  $3.07 \pm 0.62 \mu\text{m}$  (mean  $\pm$  SD,  $n = 8$ ) in diameter. The quantitative data revealed that the LD size was closely correlated with composition of LD-associated protein: If the size of LDs contained in SVCs is more than approximately  $1 \mu\text{m}$  in diameter, the LDs are coated with perilipin as well as ADRP. However, if the LDs size exceeds approximately  $2.4 \mu\text{m}$  in diameter, the LDs are completely coated with perilipin but are not (or only slightly) coated with ADRP, indicating that the SVCs have almost completed transition of LD-associated protein from ADRP to perilipin.

In the immunofluorescence observations 62 and 87 h after the inoculation, we focused on the adipocytes showing morphological characteristics that were expected to appear in the intermediate stages of the cell division. Figure 6B shows immunofluorescence images of ADRP, perilipin, and cell nucleus in such an adipocyte and its corresponding DIC image (87 h after the inoculation). In an adipocyte, indicated by arrowhead in Fig .6B, perilipin was strongly localized on the surface of LDs, whereas the ADRP was only faintly localized on the LDs. These images suggest that the transition of LD-associated proteins is almost completed. The most significant result is that DNA stained with Hoechst 33342 was localized in two regions of the adipocyte, suggesting that the adipocyte is probably in a cell division phase. Since the dividing cell became thicker than other cells, it was difficult to clearly observe both the dividing cell and other cells in the same focal plane. Hence cells, other than that indicated, appear out of focus in Fig. 6B. We also found that several adipocytes having LDs coated with only ADRP or those coated with both ADRP and perilipin were probably in a cell division phase (62 h after the inoculation; data not shown). These findings indicated that adipocytes retained the ability of cell division before and even after the transition of LD-associated proteins was almost completed.

## DISCUSSION

A confluent monolayer never formed while SVCs were differentiating into adipocytes (Fig. 1). When SVCs started accumulating LDs, they continued to exhibit a fibroblast-like shape and to show movement on the dish. The finding that contact inhibition (growth arrest) is not required for the induction of adipocyte differentiation from SVCs derived from mesenteric adipose tissues contradicts previous findings in SVCs derived from epididymal and perirenal adipose tissues of rats

(3, 4, 6) or in 3T3-L1 cells (2). We propose that this contradiction is due to either of the two possibilities. (i) Our time-lapse observations, with highly sophisticated imaging techniques, enable a more exact description of asynchronous behavior of primary SVCs. (ii) The finding that contact inhibition is not required may be a specific characteristic of SVCs derived from mesenteric adipose tissue, although similar observations for SVCs derived from other tissues or 3T3-L1 cells are yet to be verified.

Numerous small LDs (a few micrometers in diameter) were accumulated in the perinuclear region at an early stage of adipocyte differentiation (Fig. 2). Figure 4 (also Movie 3) showed that nascent LDs (several 100 nm in diameter) developed in the lamellipodia; subsequently, some of them grew into small LDs while moving toward the perinuclear region. The centripetal movements of LDs are consistent with those in 3T3-L1 cells reported previously (26). At a late stage of adipocyte differentiation, several small LDs grew to more than 10  $\mu\text{m}$  in diameter (large LDs) while the number of LDs was drastically decreasing (Fig. 2). According to these results, growth of LDs is probably achieved by the successive fusion of LDs with each other. In Movie 3, several nascent LDs appear to fuse with each other because one of two adjacent LDs vanished suddenly. However, the LDs might have vanished due to the vertical movements of the LDs in the cell. Thus, three-dimensional observation must be carried out to clearly detect the fusion event between the two LDs.

We have developed a method for the fluorescence staining of LDs in living adipocytes (Fig. 3). In previous reports (23, 25, 29), LDs in fixed adipocytes were stained with BODIPY 493/503. On the other hand, our method does not require cell fixation, and thus temporal and spatial variations of LDs can be directly assessed in living adipocytes. We confirmed that the development and movement of nascent LDs were observed in both stained (Fig. 4) and unstained adipocytes. However,

when fluorescence imaging at 5-min intervals continued for a day, the stained adipocytes in the illuminated region tended to acquire a spherical shape and detach from the dish. This suggests that the cells would be slightly affected by excitation and/or emission light. Lengthening the time intervals between the acquisitions of fluorescence images would achieve further long-term fluorescence imaging.

The most significant finding in the present study is that 12 of 48 SVCs underwent cell division even after they accumulated numerous LDs (Fig. 5) because such cell division implies that the “final” cell division during adipocyte differentiation occurs considerably later than that characterized in 3T3-L1 cells. Figure 6A and the quantitative data clearly show that SVCs expressed perilipin by 72 h after the inoculation and the transition of LD-associated proteins from ADRP to perilipin was almost completed by 87 h after the inoculation. The temporal variation of perilipin in SVCs during adipocyte differentiation is consistent with those in 3T3-L1 cells reported previously (17). In 3T3-L1 cells, the presence of perilipin-coated LDs would imply that the transcription factor peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) has been activated since PPAR- $\gamma$  is a primary regulator of perilipin expression (31). The expression of PPAR- $\gamma$  was reported to be sufficient to induce growth arrest (32; for review, see ref. 10). In contrast, when we examined the timing of adipocyte division in our time-lapse observations, it was observed that 9 of the 12 adipocyte divisions occurred after 72 h, that is, after almost all adipocytes expressed perilipin. Following this, 3 of the 9 adipocyte divisions occurred after 87 h, that is, after all LDs were coated with almost only perilipin. Moreover, Fig. 6B showed directly that SVCs were capable of division even after all LDs were completely coated with perilipin. These findings implied that the PPAR- $\gamma$  expression was not sufficient to inhibit cell division of SVCs, and thereby the timing of perilipin expression would have no correlation with that of cell division.

We further examined the relation between the LD size and the timing of cell division. As described earlier, the LD size was closely correlated to the expression of LD-associated proteins, whereas the timing of perilipin expression would have no correlation with that of cell division. These findings suggest that the LD size has also no correlation with the timing of cell division. In fact, there was no distinct difference in the size and the number of LDs between 12 of 48 adipocytes that divided during adipocyte differentiation and the others that did not. Thus, formation and subsequent growth of LDs and cell division, which play critical roles in adipocyte differentiation, would be independent or simultaneous processes rather than sequential processes, or the behavior of primary SVCs would be more asynchronous than that of 3T3-L1 cells during adipocyte differentiation.

In the process of adipocyte division shown in Fig. 5, LDs that accumulated in a parent adipocyte were evenly distributed between two daughter cells without fusing or dividing. According to a previous report (11), primary unilocular adipocytes isolated from adipose tissues can undergo division by either of the two manners. (i) In locus-dividing cell division, a single LD breaks down into numerous fine LDs, after which adipocytes divide and they evenly distribute the fine LDs between two daughter cells. (ii) In locus-preserving cell division, a fibroblast-like cell that has only a small number of LDs divides from a unilocular adipocyte; in other words, one daughter cell inherits a large single LD. The cell division of SVCs during adipocyte differentiation would correspond to the locus-dividing cell division of unilocular adipocytes rather than the locus-preserving cell division. In a previous report (33), it was observed that primary unilocular adipocytes regained fibroblast-like shapes with no LDs, that is, it underwent de-differentiation. During adipocyte differentiation, on the other hand, we never observed the morphological conversion of immature adipocytes from a spherical shape with LDs to a fibroblast-like shape with no LDs.

In conclusion, our time-lapse observations have provided a detailed account of the morphological changes that SVCs undergo during adipocyte division and differentiation. We revealed temporal and spatial variations of LDs in individual cells during adipocyte differentiation, and then observed that adipocytes divided and they distributed LDs evenly between two daughter cells. Immunofluorescence observations of LD-associated proteins strongly suggest that the “final” cell division during adipocyte differentiation occurs considerably later than that characterized in 3T3-L1 cells. We also developed a method for the fluorescence staining of LDs in living adipocytes. Fluorescence observations using confocal laser scanning microscopy or total internal reflection fluorescence microscopy would clarify further details on temporal and spatial variations of LDs, and it might detect the fusion event occurring between two LDs in living adipocytes.

#### SUPPLEMENTAL DATA

The online version of this article (available at <http://www.jlr.org>) contains supplemental movies. Movies 1, 2, 3, and 4 correspond to time-lapse observations shown in Figs. 1, 2, 4, and 5, respectively.

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## Figure legends

### **Fig. 1.**

Time-lapse phase-contrast micrographs of stromal-vascular cells (SVCs). The number in each image represents the elapsed time (h) from the start of the observation. Time 0 h corresponds to 3 days after inoculation of the cells. Some cells, indicated by arrows, had well-defined lipid droplets (LDs) at 0 h, thereby implying that they had already begun differentiating into adipocytes. Most of the other cells differentiated into adipocytes containing well-defined LDs during the observation period. The LDs accumulated in each adipocyte grew from a few  $\mu\text{m}$  to more than 10  $\mu\text{m}$  in diameter with time. Differentiated adipocytes tended to aggregate to each other (arrowhead). Bar, 50  $\mu\text{m}$ .

### **Fig. 2.**

Time-lapse differential interference contrast (DIC) micrographs showing temporal and spatial variations of LDs during adipocyte differentiation. The number in each image represents the elapsed time (h) from the start of observation. Arrowheads indicate an identical cell. Small LDs (a few  $\mu\text{m}$  in diameter) appeared in the perinuclear region; subsequently, the number of small LDs increased (0–8 h). A change in the cells was observed—from containing smaller and numerous LDs to containing larger and fewer ones (8–24 h). Some LDs grew up to 9  $\mu\text{m}$  in diameter at 24 h. After 24 h, the larger LDs continued to grow with time. Bar, 50  $\mu\text{m}$ .

### **Fig. 3.**

(A) Epifluorescence micrograph of LDs and nuclei in living adipocytes. In the micrograph, *green*

and *blue* indicates LDs stained with BODIPY 493/503 and nuclei stained with Hoechst 33342, respectively. (B) DIC micrograph corresponding to the epifluorescence micrograph. LDs (less than 1  $\mu\text{m}$  in diameter) were distributed diffusely in the cell body (arrowhead) or were aggregated in the perinuclear region (arrow). These micrographs were taken 4.5 days after inoculation. Bar, 20  $\mu\text{m}$ .

**Fig 4.**

Temporal and spatial variations of LDs observed at higher magnification. In each image, the epifluorescence micrographs of LDs (*green*) and nuclei (*blue*) are merged; subsequently, are overlaid on the corresponding DIC micrograph. The number in each image represents the elapsed time (min) from the start of observation. Time 0 min corresponds to 7.5 days after inoculation of the cells. Nascent LDs, indicated by arrowheads, gradually grew to a few  $\mu\text{m}$  in diameter while moving toward the lamellipodium/cell body transition zone (arrowheads in 30–150 min). Bar, 10  $\mu\text{m}$ .

**Fig. 5.**

Time-lapse DIC micrographs showing cell division during adipocyte differentiation. The number in each image represents the elapsed time (min) from the start of observation. Arrowheads indicate an identical parent cell or daughter cells. The adipocyte accumulating many LDs suddenly became rounded (30–60 min) and then divided into two daughter cells (60–90 min). The daughter cells can be also regarded as adipocytes because they were containing many LDs even immediately after cell division. Bar, 50  $\mu\text{m}$ .

**Fig. 6.**

Immunofluorescence micrographs of adipose differentiation-related protein (ADRP) and perilipin

through the time course of adipocyte differentiation from SVCs. Cells were fixed and stained at various time points after the inoculation. The left and middle panels show fluorescence images of ADRP (*green*) and perilipin (*red*). The right panels show the merging of two fluorescence images of ADRP and perilipin. The merged images were also overlaid on the corresponding DIC images. Bars, 20  $\mu\text{m}$ . (A) The number in each ADRP image represents the time point of fixation (h); thus, it corresponds to the duration of cell culture. Perilipin and ADRP were not clearly detected in the cells at 36 and 87 h, respectively. At 62 h, three types of LDs were observed: LDs contained mainly either ADRP (green arrowhead), perilipin (red arrowhead), or both (yellow arrowhead). (B) In the perilipin and the merged images, cell nuclei also are shown (*blue*). Perilipin-coated LDs, which are several  $\mu\text{m}$  in diameter, were accumulated in an adipocyte (arrowhead). The adipocyte nucleus appeared to be splitting into two, thereby suggesting that the cell was probably in a cell division phase. Since the dividing cell was thicker than the other cells, both the dividing cell and other cells could not be clearly observed in the same focal plane; hence, the dividing cell is in focus, while other cells appear to be out of focus. The cells were fixed and stained 87 h after the inoculation.

Figure 1

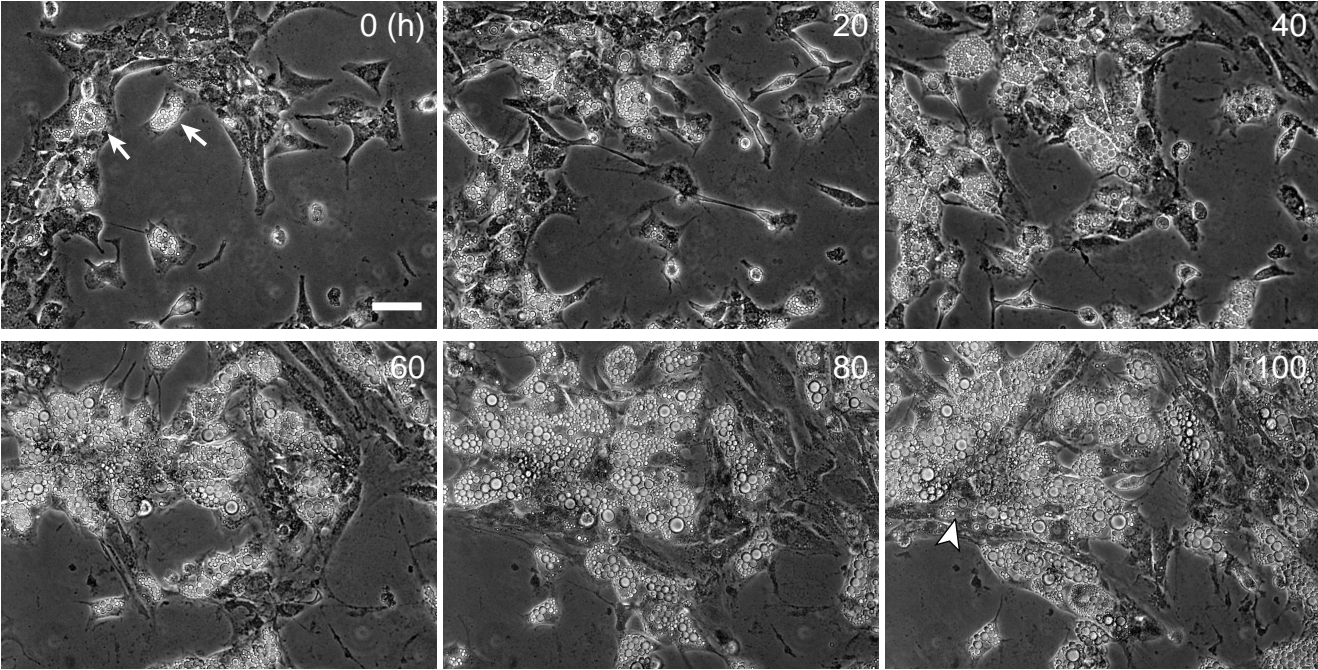


Figure 2

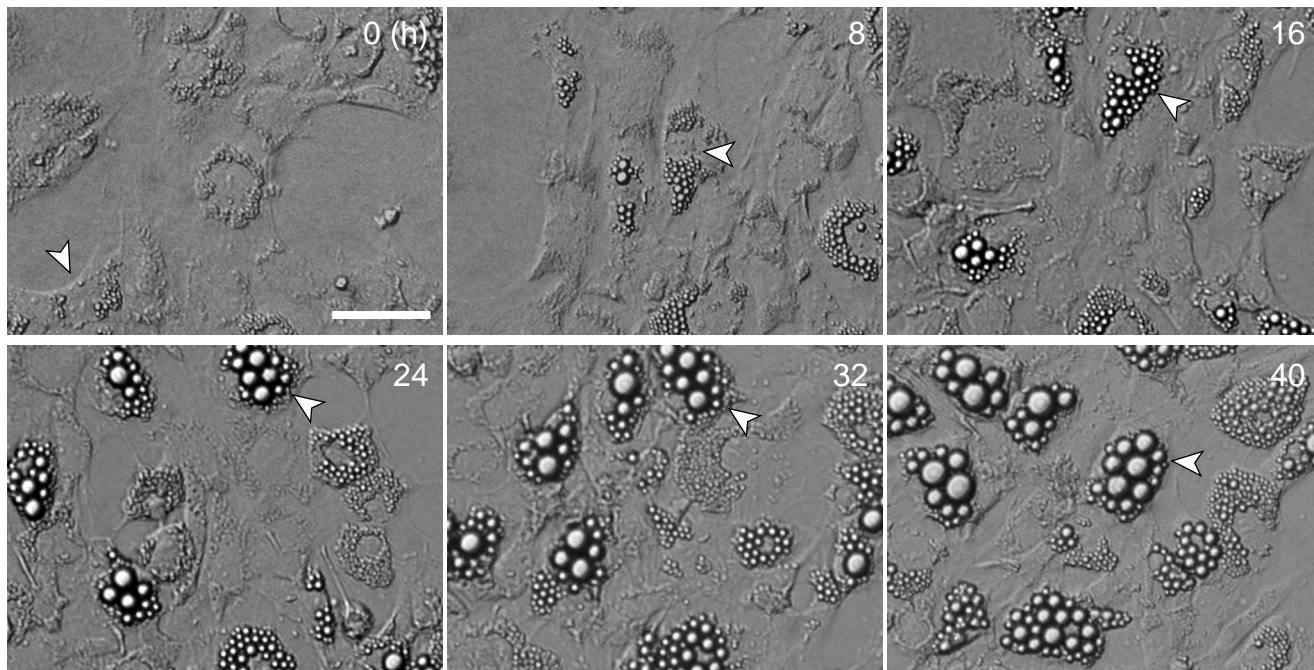


Figure 3

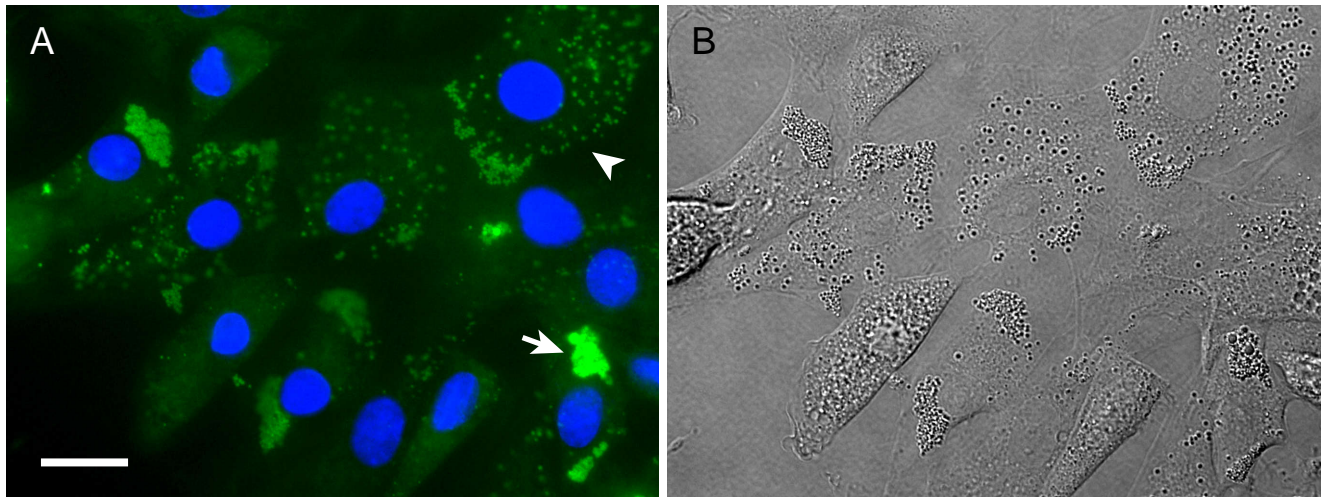


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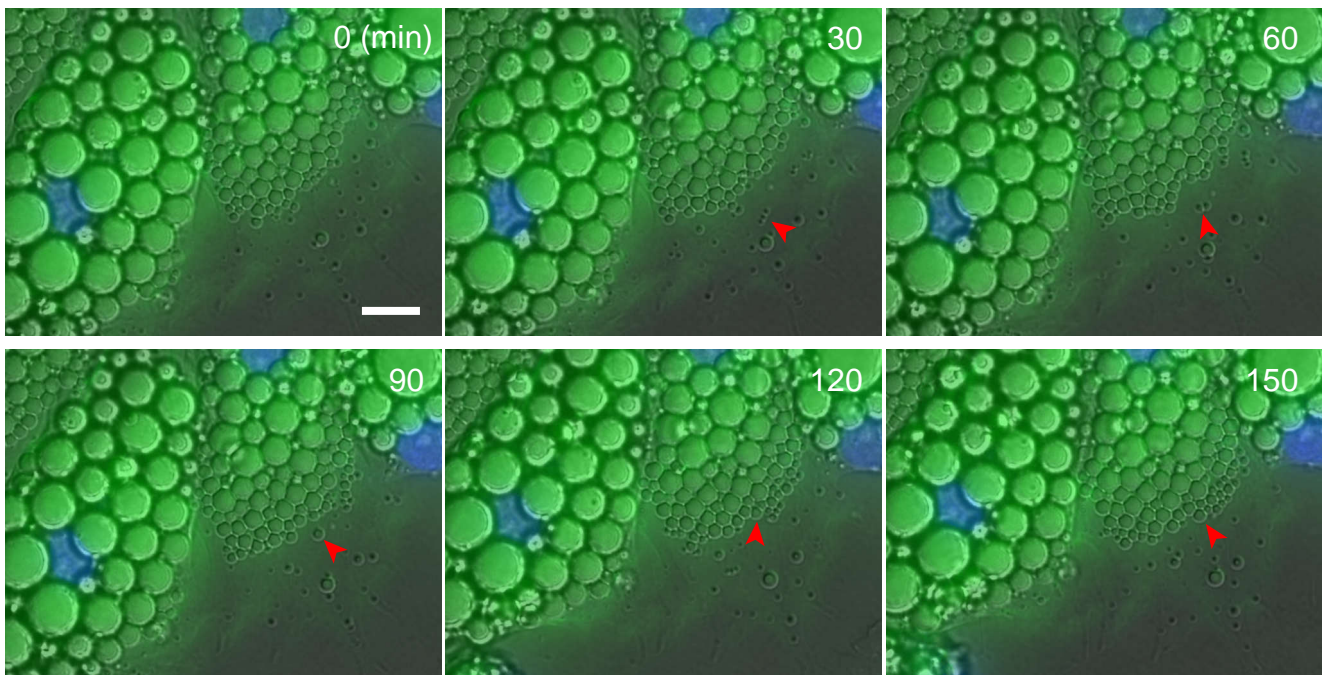


Figure 5

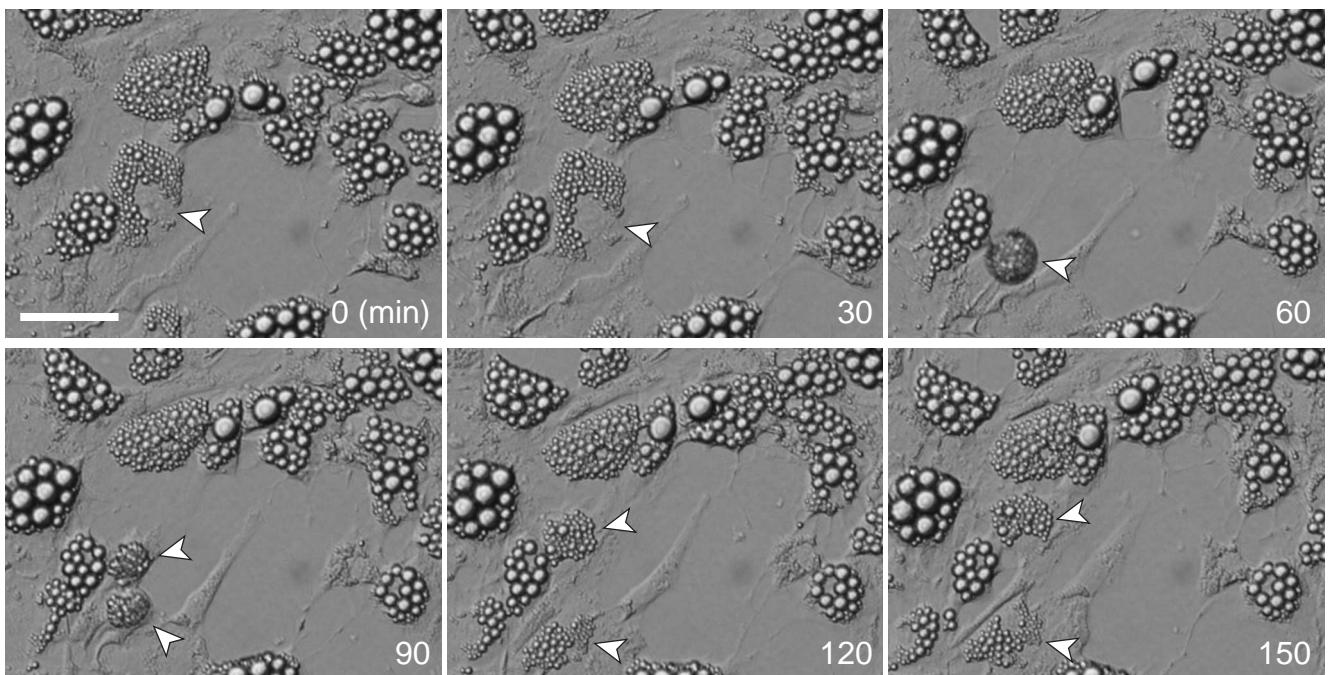


Figure 6

