methods

Simple flow cytometric method used to assess lipid accumulation in fat cells

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Abstract **Adipogenesis of preadipocytes in culture has been frequently used to study the molecular basis and effect of drugs on fat cell conversion. However, after adipogenic induction, cells respond to the inducing agent with various speeds of conversion and fat accumulation, which complicates direct molecular and biochemical analyses. Here we present a simple and sensitive method to detect and quantify fat accumulation inside cells by flow cytometry. Using this method, we detected elevated levels of cytoplasmic granularity that correlated well with an increased level of fat accumulated inside cells after adipogenic conversion. We further demonstrated the ability of this method to monitor and quantify fat cell maturation within a complex population of cells and to identify and collect the fat cells with similar fat storage for further analysis. Flow cytometry offers distinct advantages over existing detection systems for cytoplasmic lipid staining and lipid extraction and could represent a powerful analytical tool to monitor the effect of chemicals and biological molecules on fat cell conversion and maturation. Moreover, in combination with a cell sorting facility, our method offers a simple and efficient means of collecting fat cells of specific status for further analysis.**—Lee, Y-H., S-Y. Chen, R. J. Wiesner, and Y-F. Huang. **Simple flow cytometric method used to assess lipid accumulation in fat cells.** ***J. Lipid Res.*** **2004.** 45: **1162–1167.**

Supplementary key words flow cytometry • adipogenesis • fat accumulation • cytoplasmic granularity • adipocyte

The prevalence of obesity and obesity-related disorders, such as diabetes and arthritis, has promoted much research in recent years into the prevention and treatment of obesity (1–3). Understanding the molecular mechanism underlying fat cell development has been one of the major focuses of many researchers in the field. Methods in use for the assessment of fat cell development and maturation after initiation of adipogenesis in cell culture include microscopic examination of cellular lipid droplet formation and cellular lipid staining by Oil Red O (4). These methods, although excellent in detecting the presence of intracellular fat, are ineffective in objectively quantifying the degree of fat accumulation if not used in conjunction with other extraction and analytic systems. Particularly, during the process of adipogenesis of preadipocytes, it is observable that cells are heterogeneous in their response to adipogenic agents in terms of speed of adipogenic conversion and degree of fat accumulation (5). This heterogeneity of response may cause difficulties when evaluating and comparing the effects of several treatments that target only fat cells of certain status due to the masking influence of those nontargeted cells. Thus, an effective method to detect and classify cells with similar fat content will certainly increase analytical precision in monitoring fat cell development and the ability to quantify the effects of therapeutic agents. Currently, flow cytometry and fluorescence-activated cell sorters (FACSs) are extensively used in the analysis of hematological cells (6, 7); we have explored the feasibility of using these methods to assess fat content during fat cell development and to group fat cells with similar fat content for more precise biochemical and molecular analyses.

METHODS

3T3-L1 cell culture and adipogenesis

3T3-L1 preadipocytes (CL173) were obtained from American Type Culture Collection (ATCC; Manassas, VA) and maintained in culture according to the ATCC protocol. Adipogenesis was induced by adding insulin, 3-isobutyl-1-methyl-xanthine (IBMX), and dexamethasone to 2-day postconfluence cells (designated Day 0) at final concentrations of 10 μg/ml, 0.5 mM, and 1 μM, respectively, for 2 days (8). Cells were then maintained in culture media containing 5 μg/ml of insulin until needed.

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Abbreviations: ATCC, American Type Culture Collection; FACS, fluorescence-activated cell sorter; FSC, forward scatter; GFP, green fluorescence protein; IBMX, 3-isobutyl-1-methyl-xanthine; SSC, side scatter.

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Flow cytometry

Adipogenically-induced 3T3-L1 cells were analyzed and sorted in flow cytofluorometers (FACS calibur and FACS StarPlus, Becton Dickinson) using FACS technology. Cells were briefly rinsed twice with prewarmed 0.25% trypsin-EDTA and then incubated for 5 min at 37°C. Cells were then gently resuspended in PBS, washed twice with PBS, resuspended in cold PBS, and kept on ice prior to flow cytometric analysis. For cell sorting, the sorted cells were collected directly into the culture medium and returned to the CO_2 incubator immediately after sorting. The cytofluorometer settings for both side scatters (SSC) and forward scatters (FSC) to analyze fat cells were dependent on the analytic sensitivity of the machine. In general, the voltages and compensation between scatters were set to the degree so that the majority of control cells (Adipo 0D, noninduced) were, as shown in **Fig. 1B**, located below the scale of 200 for the SSC and between the scales of 200 and 800 for the FSC.

RNA extraction and Northern blot analysis

Cells were homogenized in TRIzol RNA reagent (GIBCO-BRL), and total RNA was isolated according to the manufacturer's protocol. Total RNA (5 μg) was denatured, electropho-

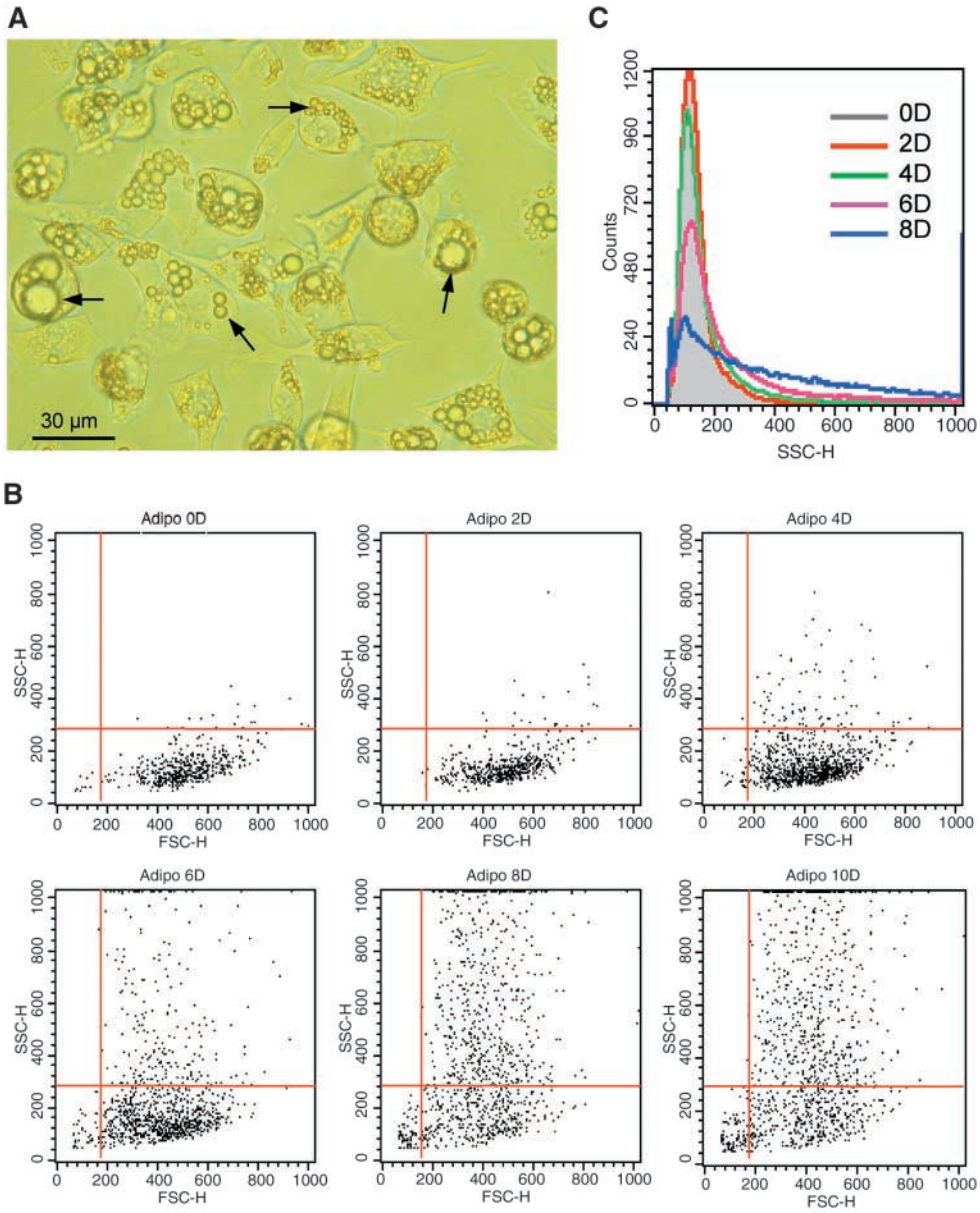

Fig. 1. Increased granularity and heterogeneity among 3T3-L1 cells during adipogenesis. A: Light microscopic examination of 3T3-L1 cells 8 days after adipogenic induction. Arrowhead indicates the lipid droplets accumulated inside cells. B: Dot plots of side scatter (SSC; *x* axis) versus forward scatter (FSC; *y* axis) of 3T3-L1 cells generated from flow cytometric analysis of levels of granularity at different time points of adipogenesis. FSC represents the cell size, whereas SSC represents cytoplasmic granular intensity. Two-day post confluent 3T3-L1 cells (designated Day 0, shown here as Adipo 0D) were hormonally induced for adipogenesis. Cells were dissociated and analyzed in a flow cytometer at 0, 2, 4, 6, 8, and 10 days after induction. C: The overlay of cell distribution versus cellular granular intensity from five plots in 1B.

resed, transferred to a nylon membrane, and probed with a ^{32}P-labeled perilipin cDNA probe using standard protocols.

Oxygen consumption

Immediately prior to measurement, cells were harvested by trypsinization, washed twice in PBS, and resuspended in 0.2 ml PBS. Cells were then added to an Oxytherm oxygen electrode (HansaTech, UK) previously equilibrated and stabilized with air in a 0.5 ml oxygen buffer at 37°C. Oxygen consumption was monitored for 10 min and calculated according to the manufacturer's instructions. The electrode buffer contained 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4, and 1 mg/ml BSA (pH 7.4).

Additional protocols

Mouse preadipocytes were isolated from male epididymal fat pads and cultured accordingly (9). Adipogenesis induction for the isolated preadipoctes was similar to that for 3T3-L1 cells. In addition, BRL49653C (Rosiglitazone, Smithkline Beecham, UK) was included in the induction media. To measure cellular lipid, cells were briefly homogenized in PBS using a Teflon homogenizer. After removal of cell debris, lipid levels were measured using a triglyceride detection kit (Infinity, Sigma). For cytoplasmic lipid staining, cells were fixed in 70% isopropanol and stained in 0.2% Oil Red O (4). Recombinant adenovirus carrying the green fluorescence protein (GFP) marker gene, and a gene of interest was generated using the AdEasy vector system (Q-BIOgene).

RESULTS AND DISCUSSION

3T3-L1 cells, grown to 2-day postconfluence in culture (designated Day 0, shown as Adipo 0D in Fig. 1), were

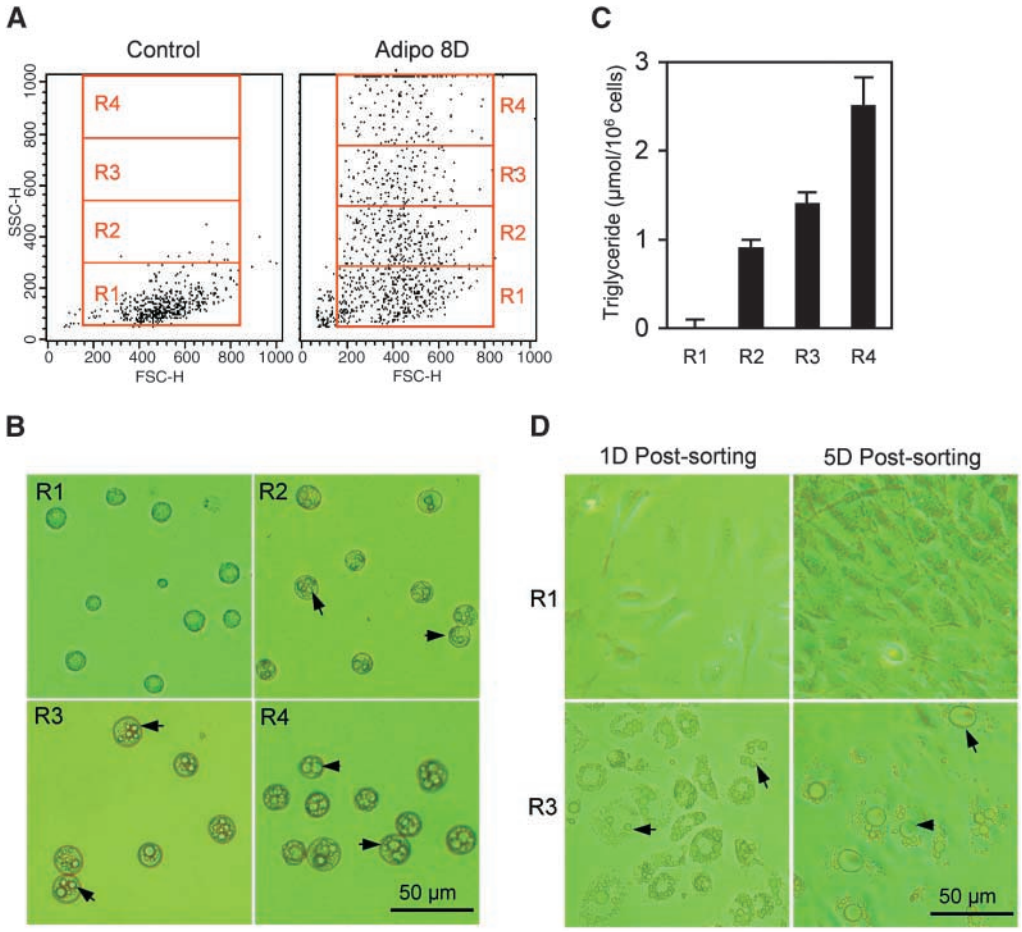

Fig. 2. Positive correlation between the cellular lipid content and granularity of 3T3-L1 cells after adipogenic induction. A: The regions gated for cell sorting. Scatter plot of 3T3-L1 cells 8 days after adipogenic induction was gated into four regions based on their granularity distribution. The R1 region was gated to include the majority of control cells shown on the SSC scale. Regions R2 to R4 were gated to include the remaining range of SSC, and each region contained an equal range of SSC. Cells from these four regions were then collected separately. The *x* axis, FSC, represents the cell size, whereas the *y* axis, SSC, represents cytoplasmic granular intensity. B: Light microscopy of the cultured 3T3-L1 cells collected from each region in A. Arrowheads indicate lipid droplets accumulated inside cells. C: Comparison of the cellular lipid content of cells collected from each region in A. Cells were briefly homogenized in PBS in a Teflon homogenizer. The cytoplasmic fraction was collected and assayed for triglyceride levels. The value for each gated group is the mean of four sortings and is presented as a vertical bar in the figure. The SEM for each group is shown as a vertical line. D: Light microscopy of the sorted 3T3-L1 cells from regions R1 and R3. After sorting, cells were collected from each region and recultured. Cell photographs were taken 1 day (1D) and 5 days (5D) after reculturing. Arrowheads indicate lipid droplets accumulated inside cells.

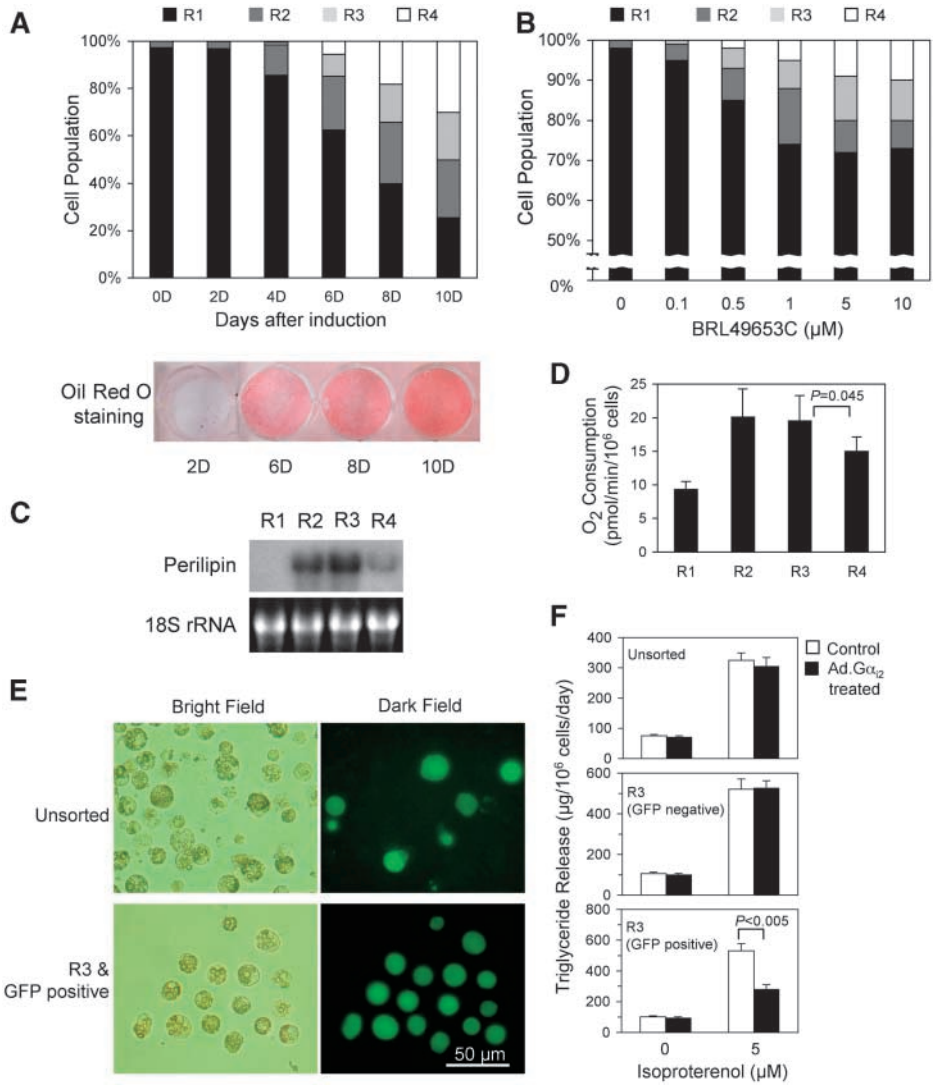

Fig. 3. Use of flow cytometry in analyzing fat cell development. A: Quantification of fat cells during adipogenesis. Two-day postconfluent 3T3-L1 cells (designated 0D) were induced for adipogenesis and analyzed at different time points for both cytoplasmic granularity in flow cytometry (upper panel) and lipid storage via Oil Red O staining (lower panel). For flow cytometric analysis, the value of granularity for a gated region at each time point was the mean of four samples, and the granularity (gated region) values for the same time point are displayed in a stacked column. B: Analysis of the dosage effect of BRL49653C on adipogenesis in isolated mouse preadipocytes. Mouse preadipocytes isolated from male epididymal fat pads were induced for adipogenesis 2 days postconfluence. Various concentrations of BRL49653C, as indicated, were added to the inducing medium. Eight days after induction, cells were analyzed for their scatter profile using flow cytometry. The granularity value for each gated region at each concentration of BRL49653C was the mean of four samples, and the gated region values at the same time point are displayed in a stacked column. C: Comparison of the levels of perilipin mRNA in cells from each region. Five micrograms of total RNA from cells were electrophoresed, blotted onto a nylon membrane, and probed with a ^{32}P-labeled perilipin cDNA probe. D: Comparison of oxygen consumption among fat cells gated for levels of fat storage. Four groups of cells were collected based on their SSC profile with regards to granularity, as those shown in Fig. 2A, and recultured for at least a further 12 h in culture media prior to oxygen consumption measurement. The value for each gated group is the mean of four sortings and is presented as a vertical bar in the figure. The SEM for each group is shown as a vertical line. E: Identification and collection of GFP-carrying adenovirus targeted fat cells displaying similar fat accumulation. Two days postconfluent 3T3-L1 cells were induced for adipogenesis. Four days after induction, recombinant adenoviruses carrying a GFP marker gene were added to the culture medium at the dosage of 5× infection and incubated for 24 h. Eight days after induction, cells were dissociated (upper panel) and analyzed in a flow cytometer. The cells that displayed a scatter profile in the R3 gated region (see Fig. 2A) and emitted green fluorescence were selected and collected for fluorescence microscopy (lower panel). F: Lipolytic effect of isoproterenol on fat cells infected with the adenovirus carrying the GFP marker gene and a gene encoding for $G\alpha_{i2}$. Similar to the experiment described in E, cells were infected with a recombinant adenovirus carrying the $G\alpha_{i2}$ expression cassette. Fat cells displaying an SSC profile in the R3 region and emitting green fluorescence were collected for reculture. Isoproterenol was added to the culture media 1 day after reculture. One day after the addition of isoproterenol, 100 μl of the condition media were removed for triglyceride measurement. The value for each gated group is the mean of four sortings and is presented as a vertical bar in the figure. The SEM for each group is shown as a vertical line.

hormonally induced for adipogenesis (8) then dissociated and analyzed in a flow cytometer at 0, 2, 4, 6, 8, and 10 days postinduction (Fig. 1A, B). The dot plot of cytometric FSC (shown as the *x* axis in Fig. 1B and **Fig. 2A**) and SSC (shown as the *y* axis) reflecting the cell diameter and granular structures within the cell, respectively, showed that after adipogenic induction, the cells had become increasingly heterogeneous in their cellular granularity (Fig. 1B). After adipogenic induction, the cells containing greater granular structure were markedly increased, and this increase in granularity positively correlated with the time of the postadipogenic induction (Fig. 1B, C). In general, the longer the period of induction, the greater the quantity of lipid a fat cell in culture can accumulate. Thus, to determine whether increasing the fat stored within a cell would result in the greater granularity seen in SSC, 8 days postinduction cells were gated for four regions as shown in Fig. 2A, based on the full scale of SSC. The R1 region contains cells with a level of granularity similar to that seen in the control cells (noninduced), whereas R2 to R4 regions contain cells with increasing granularity. Cells in the gated regions were sorted and collected for further microscopic examination (Fig. 2B) and lipid content analysis (Fig. 2C). Indeed, both microscopic and lipid analyses of the sorted fat cells revealed that the granularity detected in the SSC correlated well with levels of fat storage within cells (Fig. 2B, C); the cells collected from the R4 region had the greatest intracellular granularity with the biggest lipid droplets and highest lipid content, while cells that exhibited lower granularity (R2, R3) contained fewer and smaller lipid droplets and a lower lipid content (Fig. 2B). After sorting, cells collected from all regions were analyzed for their ability to reattach to the surface of culture dish under culturing conditions (Fig. 2D). It was found that the degree of reattachment was negatively correlated with the increased cellular granularity; more than 95% of the cells from the R1 region could reattach, while less than 10% of cells from the R4 region could reattach. Cells in the R1 region appeared not to be induced and could proliferate, while cells from other regions continued to accumulate fat as indicated by the growth of their cellular lipid pool (Fig. 2D). Nevertheless, our results indicate that the granularity of fat cells, as detected by flow cytometry, is an excellent indicator for stored cellular fat, and thus, the granularity of a fat cell can be easily distinguished via flow cytometric SSC.

Quantification of distinct cell populations is one of the integral functions of flow cytometry. Thus, we next used flow cytometry to monitor and quantify 3T3-L1 cells based on their degree of fat accumulation at various stages of adipogenesis. **Figure 3A** summarizes and compares the R1, R2, R3, and R4 cell populations of 3T3-L1 cells at various stages of adipogenesis. Changes in cell distribution from low to high granularity during the 10-day period of adipogenesis were clearly profiled by flow cytometry (Fig. 3A, upper panel). In comparison, cells at various stages of adipogenesis were also stained with Oil Red O, which has been routinely used to monitor adipogenesis. Although the Oil Red O staining clearly indicated the presence of lipid droplets within cells, by itself it did not provide any quantitative information regarding the lipid levels or the heterogeneity of fat accumulation among cells, unlike flow cytometry (Fig 3A, lower panel). Accordingly, we hypothesized that flow cytometry also might be more sensitive in detecting fat content when used to evaluate and compare the effect of different factors on fat accumulation in fat cells. To examine this possibility, we isolated preadipocytes from mouse epididymal fat pads and maintained them in culture for adipogenic conversion. To induce these cells to become fat cells, a PPARγ ligand, BRL49653C (10), was needed in addition to the insulin, IBMX, and dexamethasone cocktail that were adequate to adipogenically induce 3T3-L1 cells. Thus, these isolated preadipocytes are ideal for use in evaluating the effect of PPARγ ligands on adipogenic conversion. To evaluate the dosage effect of BRL49653C on adipogenic conversion, isolated preadipocytes were cultured to confluency and induced for adipogenesis utilizing the same system used for 3T3-L1 cells, except that BRL49653C was included in the inducing media. Eight days after induction, cells were analyzed for their granularity using flow cytometry (Fig. 3B). At concentrations of 0.5 μM and higher, BRL49653C significantly increased the cell populations in the R2 to R4 regions, where cells contain greater granularity, suggesting that more cells were adipogenically induced to store fat. Although at the concentration of 5 μM BRL49653 did not further enhance the total level of adipogenesis (R2 to R4), the population of cells containing greater granularity (R4) increased relative to the cell populations from the R2 and R3 regions (Fig. 3B).

As demonstrated above, despite being under the same culturing condition, cells underwent adipogenesis at varying rates reflected by the degree of their fat accumulation. We were, thus, interested to see whether these fat cells with various degrees of fat storage differed in other physiological parameters associated or influenced by their cellular fat content. To test this, we analyzed and compared the expression levels of perilipin, a lipid droplet-specific protein that coats the surfaces of lipid droplets of adipocytes (11), and the oxygen consumption of fat cells with different levels of fat storage (the R2 to R4 regions). 3T3-L1 cells, 8 days after adipogenic induction, were sorted and collected based on their granularity, similar to those cells shown in Fig. 2A. The collected cells were monitored for their perilipin mRNA levels and their oxygen consumption (Fig. 3C, D). We found that cells in the R1 region did not express perilipin mRNA and consumed the least oxygen relative to the cells having higher granularity (R2 to R4). In addition, levels of perilipin mRNA, in cells from the R2 and R3 regions, were found to be higher than in cells from the R4 region. Similarly, cells from the R2 and R3 regions consumed significantly more oxygen than those from the R4 region, indicating that fat cells with less fat stored are more active in oxidation when compared to those with higher fat storage.

Finally, to precisely analyze the effect of a treatment, such as overexpression of a gene in fat cells, we used a flow cytofluorometer to identify and collect fat cells that

were carrying similar fat stores and were subjected to treatments for further analysis. Here, we used a recombinant adenoviral vector to deliver the GFP marker gene and the Gα inhibitory subunit 2 ($G\alpha_{i2}$) expression cassette into fat cells. 3T3-L1 cells, 4 days after adipogenic induction, were infected with the recombinant adenoviral vector AdTrack, carrying either the GFP marker gene under the control of a CMV promoter (12) or the recombinant adenoviral vector-carrying $G\alpha_{i2}$, in addition to the GFP marker gene. Despite the use of the highly efficient adenoviral gene delivery system and a viral dosage of over 5× infection, only a small number of fat-laden cells were found to express the GFP marker gene after 3 days of infection. To specifically select those fat cells that had similar fat content, we again used flow cytometric SSC in combination with fluorescence detection. As shown in Fig. 3E, a group of GFP-positive fat cells with similar fat content were effectively sorted and collected for further analysis to determine the effects of $G\alpha_{i2}$ on the lipolytic activity of fat cells stimulated by the β adrenergic receptor agonist, isoproterenol. The inhibitory effect of $G\alpha_{i2}$ on the isoproterenol-stimulated lipid release was not detected when the condition medium from the whole population of cells was analyzed (Fig. 3F). This could be due to the low infection rate of the adenoviral vector and/or the heterogeneous population of cells that contained close to 40% of the cells that failed to be adipogenically induced (Fig. 3A). However, a significant inhibitory effect of $G\alpha_{i2}$ on the isoproterenol-stimulated lipid release was measured in the condition medium from the GFP-positive cells collected in the R3 region (Fig. 3F). This result further indicates the necessity to utilize an analytical system capable of monitoring, quantifying, and selecting fat cells with different degrees of adipogenic conversion before an objective comparison among treatments can be made.

In conclusion, this study demonstrates the feasibility of directly measuring the fat content of specific cells in a complex population of fat cells using flow cytometry. Not only is the system simple, sensitive, and quantitative, but it also offers an advantage over the existing methodology, with its capability of monitoring the degree of intracellular fat accumulation in a precise, fast, and selective manner. These results provide a basis for developing a variety of applications that include diagnostic and drug screening procedures to monitor fat cell development.

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