Establishment of The 3T3-L1 Adipocyte Model for Investigation of GLUT4 Translocation

Thanarat Salahong[1, 2, 3], Chutima Chanprasert[2, 3], Ladawan Khowawisetsut[4], Pa-thai Yenchitsomanus[2, 3], Nattachet Plengvidhya[3, 5], and Prapaporn Jungtrakoon Thamtarana[*, 2, 3]

[1]Graduate Program in Immunology, Department of Immunology,
Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[2]Cellular and Molecular Biology of Diabetes Research Group, Division of Molecular Medicine, Research Department,
Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[3]Siriraj Center of Research Excellence for Diabetes and Obesity,
Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[4]Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[5]Division of Endocrinology and Metabolism, Department of Medicine,
Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[*]Corresponding author, E-mail: prapaporn.jun@mahidol.ac.th

Abstract

The translocation of glucose transporter type 4 (GLUT4) containing vesicles from cytoplasm to plasma membrane in response to insulin stimulus is crucial for maintenance of glucose homeostasis. Failure in transporting GLUT4 to plasma membrane hinders peripheral glucose uptake and, thus, leads to hyperglycemia. Here, by tagging of GLUT4 extracellular domain with hemagglutinin (HA) epitope, we aim to establish an adipocyte model for investigation of GLUT4 translocation. Lentiviral particles carrying GLUT4-HA was generated and transduced into 3T3-L1 pre-adipocytes. Cells stably expressing GLUT4-HA were validated using Western blotting analysis (WB) and immunofluorescence staining assay (IFA). After differentiation to adipocyte, the level of GLUT4-HA expressed on plasma membrane was immunofluorescence-stained, without cell permeabilization, and quantified by flow cytometry. The result showed that, as determined by WB, the established pre-adipocyte model expressed the recombinant GLUT4-HA. IFA by skipping the process of cell permeabilization successfully demonstrated GLUT4-HA expressed on cell surface. However, once the pre-adipocytes were undergone differentiation process, the percentage of mature adipocytes, as determined by cellular size and granularity using flow cytometry, did not conform to what was observed under inverted microscopy. The fluorescent intensity of the immunostained adipocytes under basal and insulin-stimulated conditions was not different. Adipocyte-specific marker should be applied for discriminating the differentiated cells by means of flow cytometry before validating insulin-stimulated GLUT4 translocation in the established model.

Keywords**:** *adipocytes, GLUT4 translocation, insulin, flow cytometry*

1. Introduction

Glucose uptake through glucose transporters (GLUTs) of peripheral cells in response to insulin action is crucial for lowering of blood glucose level and maintaining glucose homeostasis. Up to date, at least 14 members are described in the GLUT protein family (Huang, & Czech, 2007; Chadt, & Al-Hasani, 2020). The fundamental structure of GLUTs comprises transmembrane regions, binding sites of glucose and ATP, and C- and N-terminus located in the cytoplasm (Mueckler, & Thorens, 2013). Among the 14 GLUTs, glucose transporter type 4 (GLUT4) is predominantly expressed in insulin-sensitive cells, especially adipocytes and muscle cells, whereby it is responsible for insulin-stimulated glucose uptake (Navale, & Paranjape, 2016). In a basal condition, only 5% of GLUT4 is expressed on the cell surface, majority of GLUT4 is retained in the intracellular membrane compartment, referred to as GLUT4 storage vesicles (GSVs). Insulin stimulates the translocation and the fusion of GSVs to plasma membrane, resulting in 10- to 30-fold increase in glucose uptake (Leney, & Tavare, 2009; Leto, & Saltiel, 2012). The defect of GLUT4 translocation leads to hyperinsulinemia and consequently causes type 2 diabetes (T2D) and metabolic syndrome in human subjects (Shulman, 2004; Shan, Chen, Zhu, Jiang, & Zhou, 2011). In adipocyte-specific GLUT4 knockout mice, insulin-stimulated glucose uptake in adipocytes is strikingly decreased. Unresponsiveness of adipocytes to

insulin stimulus indirectly induces systematic insulin resistance and eventually causes T2D development (Minokoshi, Kahn, & Kahn, 2003). Given the fact that insulin-stimulated GLUT4 translocation is crucial for glucose uptake and that insulin responsiveness in adipocytes has a systematic effect on maintenance of glucose homeostasis, investigation of insulin-stimulated GLUT4 translocation in adipocytes is one of the strategies for studying the effect of mutations on insulin sensitivity and development of T2D.

Several approaches have been developed for monitoring GLUT4 translocation in various cell models (Qu et al., 2011; Lizunov et al., 2012; Komakula, Tiwari, & Singh, 2021). The membrane fractionation combined with Western blot analysis (WB) is commonly used for the study of GLUT4 expression on plasma membrane. However, GLUT4 proteins that are located adjacent to the plasma membrane and GLUT4 proteins that are fully incorporated into the plasma membrane cannot be distinguished with this technique (Lee, Ryu, Souto, Pilch, & Jung, 1999; Lauritzen, & Schertzer, 2010). Although photoaffinity labeling followed by immunoprecipitation can overcome this problem, this method requires many complicated labeling and post-labeling steps to quantify GLUT4 protein level (Lauritzen, & Schertzer, 2010; Smith, & Collins, 2015). Quantification of GLUT4 on cell surface using flow cytometry can specifically detect GLUT4 on cell surface. However, an antibody specific to extracellular domain of GLUT4 is currently unavailable, we cannot directly immunostain GLUT4 on cell surface before quantitating by means of flow cytometry. This study aimed to generate adipocyte cells stably expressing GLUT4 with hemagglutinin (HA) epitope inserted into extracellular domain (GLUT-HA) for investigation of GLUT4 translocation by means of flow cytometry.

2. Objectives

1. To construct lentiviral transfer plasmid carrying GLUT4 with HA epitope inserted into extracellular domain (GLUT-HA)
2. To generate a line of adipocytes stably expressing the recombinant GLUT-HA by means of lentiviral gene delivery
3. To establish the system for quantitating GLUT4-HA expressed on cell surface by means of immunofluorescence staining assay (IFA) and flow cytometry

3. Materials and Methods

3.1 Cell culture

Human embryonic kidney HEK293 (ATCC number: CRL-1573) cell line was maintained in the high-glucose Dulbecco's modified eagle medium (DMEM) medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. The murine 3T3-L1 pre-adipocyte (ATCC number: CL-173) cell line was cultured in the DMEM medium supplemented with 10% heat-inactivated bovine calf serum, and 100 µg/ml streptomycin, and 100 units/ml penicillin. Cell line was maintained at 37°C with 5% CO_2 in a humidified incubator.

3.2 Construction of pLX304 vector carrying GLUT4-HA coding sequence

The coding sequence of GLUT4 was amplified from cDNA libraries of HeLa cells. The GLUT4 amplicon and the lentiviral transfer vector pLX304 (Invitrogen, #25890) were digested with 20 units of *Bam*HI and *Pac*I in 1X CutSmart buffer and incubated at 37°C for 16 hours. Then, the digested amplicon and vector were ligated by using 5 units of T4 DNA ligase in 1X T4 ligase buffer, and the reaction was carried out at 16°C for 16 hours. The ligated product was transformed into *Escherichia coli* (*E. coli*) strain Stbl3 via conventional heat shock method. Screening of positive transformants was conducted using colony PCR and validated by BTSeq™ sequencing (U2Bio Co., Ltd, Korea). The HA epitope was inserted between amino acid 67 and 68 in the first extracellular loop of GLUT4 using site-directed mutagenesis with the primers introducing HA coding sequence. The parental pLX304-GLUT4 strand was eliminated by *Dpn*I treatment. The remained plasmid carrying HA tag (pLX304-GLUT4-HA) was transformed into *E. coli* strain Stbl3. The GLUT4-HA positive clone was screened by means of colony PCR and validated by BTSeq™ sequencing.

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3.3 Generation of the 3T3-L1 pre-adipocytes overexpressing GLUT4-HA

Nine µg of pLX304-GLUT4-HA lentiviral transfer vector, 9 µg of pCMV-dR8.91 packaging vector, and 0.9 µg of VSV-G envelope expressing vector (Addgene, #14888), were co-transfected into $3.8x10^6$ HEK293 cells seeded on 10 cm dish by using Lipofectamine 2000® (Invitrogen). Supernatant was collected at 24 and 48 hours after transfection. After filtration, viral particles in supernatants were concentrated using super speed centrifugation at 20,000 g for 180 min at 4°C. The pellet of viral particles was dissolved in the pre-adipocyte medium before transduction. The 3T3-L1 pre-adipocytes were transduced with lentiviral particle delivering GLUT4-HA in the presence of 8 ug/ul polybrene. The transduced cells were undergone selection for 10 days in the presence of 10 µg/ml blasticidin. The protein expression levels of GLUT4 were determined by and Western blotting analysis and IFA. Immunofluorescent signal was visualized under confocal microscopy (LSM 510 Meta, Zeiss).

3.4 Differentiation the 3T3-L1 pre-adipocytes overexpressing GLUT4-HA to adipocytes

The transduced 3T3-L1 pre-adipocytes were cultured in the pre-adipocyte media (DMEM supplemented with 10% bovine calf serum, 100 µg/ml streptomycin, and 100 units/ml penicillin) in a CO_2 incubator for 48 hours before media refreshment. Cells were cultured for another 48 hours. Then, the cultured media was replaced with differentiation media (DMEM supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone, 2 µM rosiglitazone, and 1 µg/ml insulin) for 48 hours. These cells were further incubated with adipocyte media (DMEM supplemented with 10% FBS and 1 µg/ml insulin) for 10 days, with media refreshment every 2-3 days.

3.5 Western blotting analysis

Twenty µg of extracted protein was mixed with 5X Laemmli sample buffer and was heated at 95°C for 5 minutes. The reduced and heat-denatured protein was separated according to the size, through sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE), at 120 V, 3000 mA for 2 hours. After that, the protein on the polyacrylamide gel was transferred onto a nitrocellulose membrane via the semi-dry blotter at 85 V for 1.40 hours. The non-specific protein on membrane was blocked with 5% skim milk in 1X Tris-Buffered Saline, 0.1% Tween® 20 Detergent (TBST) buffer. Then, the membrane was incubated with the primary antibodies diluted with 5% skim milk in 1X TBST (Rabbit anti-HA, 1:5000, Abcam #ab9110; mouse anti-β-actin, 1:1000, Santacruz, #sc-47778) at 4°C for overnight. Then, membrane was incubated with horseradish peroxidase (HRP)-linked secondary antibodies (Swine anti-rabbit Ig/HRP, 1:1000, Dako, #P0217; rabbit anti-mouse Ig/HRP, 1:1000, Dako, #P0260) diluted with 5% skim milk in 1X TBST at room temperature for 2 hours. This membrane was added the ECL chemiluminescent reagent (Roche Diagnostics). The chemiluminescent signal was visualized on a biomolecular imager (ImageQuant LAS 4010, GE Healthcare Life Sciences). Finally, the ImageJ software was used for analysis of band intensity.

3.6 Immunofluorescent staining and imaging

The 3T3-L1 pre-adipocytes were seeded on the glass coverslips coated with 1% gelatin in the 6-well culture plate. Then, the cells were fixed with 0.5 ml of 4% paraformaldehyde in 1X PBS for 15 minutes at room temperature. Non-specific proteins were blocked by 500 µl of 5% FBS in 1X PBS buffer at room temperature for 15 minutes. The target protein was stained by primary antibody (Rabbit anti-HA 1:100, Abcam, #ab9110) diluted in 1X PBS buffer for 1 hour at 37°C. After removal of primary antibody, the AlexaFlour 488 donkey anti-rabbit IgG secondary antibody (1:1000, Invitrogen, #A21206) diluted in 1X PBS and Hoechst 33342 were added at ratio 1:1000 and incubated at 37°C for 30 minutes in the dark place. After mounting the coverslip with 30% glycerol, the image was taken by a Zeiss LSM 510 Meta confocal laser-scanning microscope.

3.7 Determination of insulin-stimulated GLUT4 translocation

The non-transduced 3T3-L1 pre-adipocytes and the pre-adipocytes overexpressing GLUT4-HA seeded on the 6-well culture plate were differentiated into mature adipocytes. At day 10 post-differentiation,

cells were exposed the medium with or without 170 nM insulin for 20 minutes. Then, the cells were detached by scraper. The dispersed cells were collected by centrifugation at 300 g for 5 minutes at 4°C. Non-specific proteins were blocked with 3% FBS in 1X PBS at room temperature for 30 minutes. A primary antibody (Rabbit anti-HA, 1:1000, Abcam, #ab9110) diluted in 1X PBS with 5% FBS was then added and incubated at room temperature for 1 hour before centrifugation at 1000 g for 15 seconds. The AlexaFlour 488 donkey anti-rabbit IgG secondary antibody (1:1000, Invitrogen, #A21206) diluted in 1X PBS with 3% FBS was gently mixed with the cell pellets and incubated at room temperature in the dark for 30 minutes. After incubation. Then, cell pellet was resuspended by 300 µl of 1X PBS and stored at 4°C in the dark until ready for flow cytometer analysis.

4. Results and Discussion

4.1 Results

Construction of lentiviral transfer plasmid pLX304 carrying GLUT4-HA

The map of pLX304 construct carrying GLUT4-HA was showed in Figure 1a. The pLX304 vector and the GLUT4 amplicon were cut with *Pac*I and *Bam*HI sites. As expected, the digested vector and amplicon showed the sizes of 7,760 bp, and 1,530 bp, respectively (Figure 1b). After ligation and transformation, screening for positive transformants by colony PCR, followed by electrophoresis (Figure 1c) showed that only one (clone no. 4) from a total of 13 clones carried GLUT4 coding region, with the PCR product size of 1,061 bp. Sequencing of plasmids extracted from the positive clone showed that the nucleotides were 100% identical to that of the reference GLUT4 sequence (NM001042).

The constructed pLX304-GLUT4 was used as the template for synthesizing of GLUT4-HA by site-directed mutagenesis. Colony PCR was applied for screening of clone carrying GLUT4-HA. The expected sizes for amplicons carrying GLUT4 and GLUT4-HA were 567 and 594 bp, respectively. The PCR products separated by polyacrylamide gel electrophoresis and stained by conventional silver staining method are shown in Figure 1d. Two positive clones (clones no. 1 and 5) from a total of 10 clones were obtained. Sequencing of plasmids extracted from the positive clone revealed the correct HA coding sequence (5'-TAC CCATACGATGTTCCAGATTACGCT-3') inserted in between nucleotide 201 and 202 of GLUT4 coding sequence (Figure 1e). In conclusion, lentiviral transfer vector carrying the HA peptide sequence was successfully established.

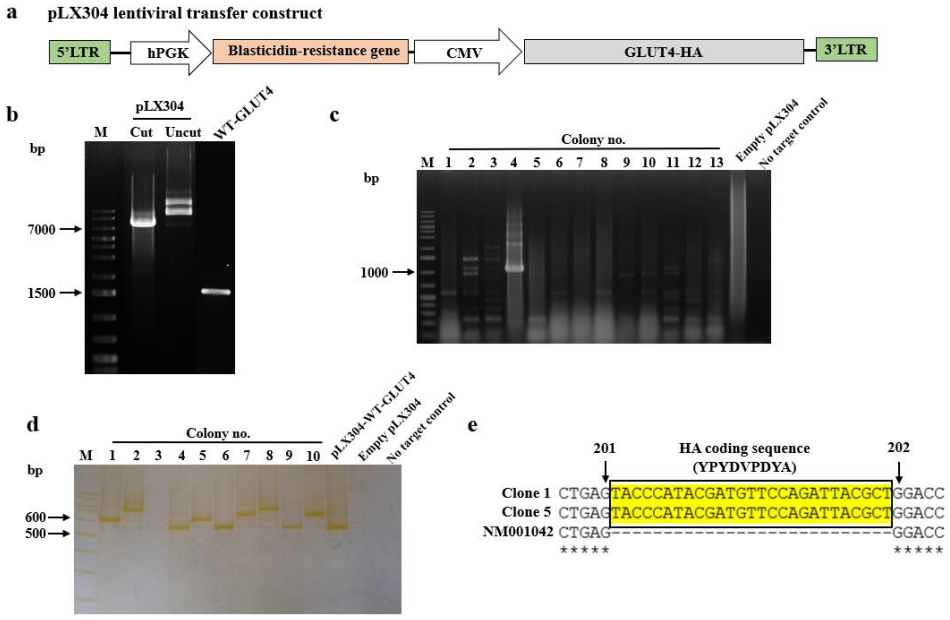

Figure 1 Construction of lentiviral transfer plasmid pLX304 carrying GLUT4-HA

Expression of the recombinant GLUT4-HA protein in the murine 3T3-L1 pre-adipocytes

The constructed plasmid pLX304 carrying GLUT4-HA, together with the packaging and envelope plasmids, were co-transfected into HEK293 cells. Viral particles generated in HEK293 cells were transduced into 3T3-L1 pre-adipocytes. Cells stably expressing GLUT4-HA were selected through blasticidin treatment. The WB analysis revealed the expression of GLUT4-HA in the transduced cells (Figure 2a). Immunofluorescence staining, without cell permeabilization, followed by confocal microscopy successfully demonstrated the GLUT4-HA on pre-adipocyte cell surface (Figure 2b).

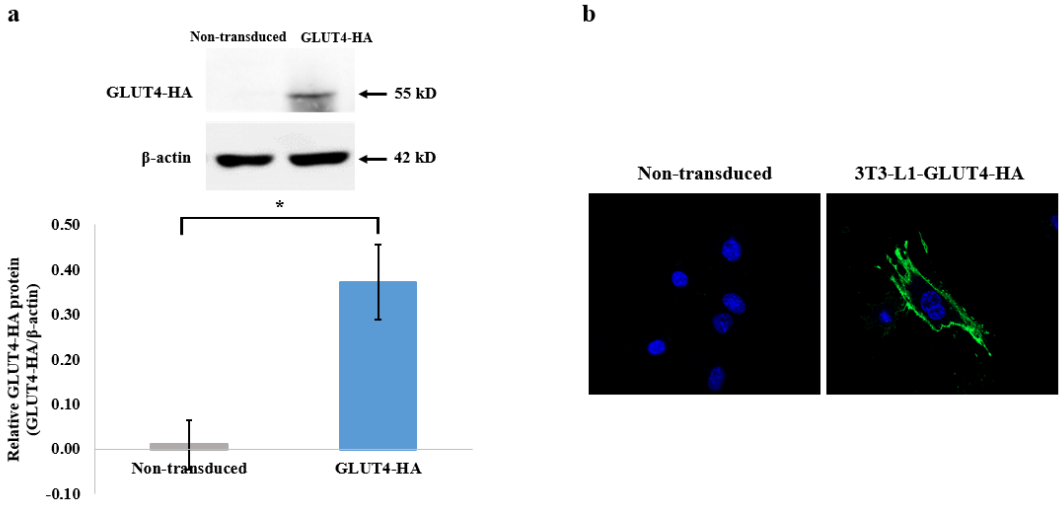

Figure 2 The recombinant GLUT4-HA protein expression in the murine 3T3-L1 pre-adipocytes

Validation of the system for quantitating GLUT4-HA expressed on cell surface

The established pre-adipocytes were allowed to differentiate into adipocyte for 10 days. As observed under inverted microscopy, the mature adipocytes were estimated at 40% of total population (Figure 3a). To quantitate GLUT4-HA expression on the adipocytes in response to insulin stimulus, cells were divided into basal and insulin-treated conditions. After 20 minute of insulin treatment, cells were detached, undergone IFA without cell permeabilization, and subjected to flow cytometry. The forward scatter (FSC) and side scatter (SSC) properties of cells were used for discrimination of mature adipocytes from pre-adipocytes. Unexpectedly, the percentage of mature adipocytes as determined by flow cytometry was only around 4%, which was not concordant with what was observed under the microscopic (Figure 3b). This result suggested that the gating strategy for discrimination of differentiated cells was not appropriate. After gating of mature adipocyte from the total cell population, it was found that the fluorescent intensities among adipocytes under basal and insulin-stimulated condition were not different (Figure 3c).

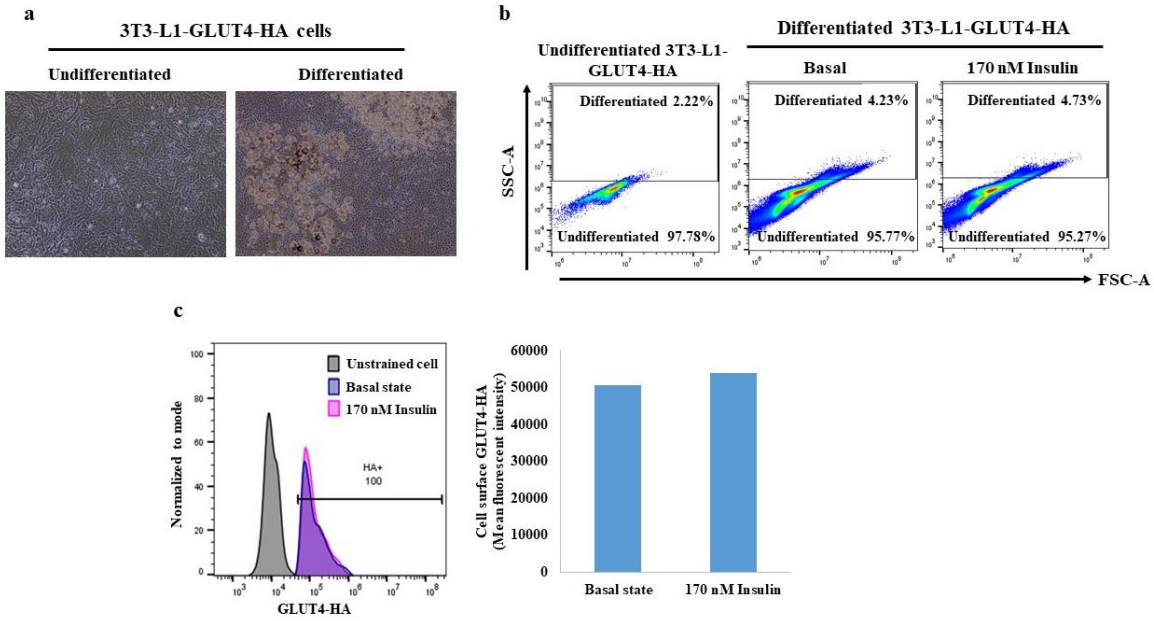

Figure 3 Validation of the adipocyte model for investigation of GLUT4 translocation

4.2 Discussion

As suggested by WB and confocal microscopic analyses, the 3T3-L1 pre-adipocytes stably expressing recombinant GLUT4-HA was successfully established and the GLUT4-HA translocated to cell surface of pre-adipocytes was detectable. Because adipocyte is one of the main insulin target cells and mature adipocytes express GLUT4 on cell surface in response to insulin stimulus greater than pre-adipocyte 10-20-fold (Govers, Coster, & Jame, 2004), inducing of adipocyte differentiation was conducted before studying of insulin-stimulated GLUT4 translocation. The current differentiation protocol cannot induce 100% cell differentiation, it is, therefore, necessary to separate mature adipocytes from undifferentiated cells, to avoid the confounding factor. Given the fact that adipocyte is spherical in shape and stores a large lipid droplet, FFC vs SSC gating was applied for separation of adipocytes from undifferentiated pre-adipocytes using flow cytometry (Lee, Chen, Wiesner, & Huang, 2004). However, the result generated from the current flow cytometry set up did not correspond to what was observed under inverted microscopy, suggesting that the methodology for cell discrimination was needed to be revised. Failure in separation of adipocytes from pre-adipocyte is a possible cause of the similarity of fluorescent intensities generated from GLUT-HA on cell surface of insulin-stimulated and unstimulated adipocytes.

Hagberg et al. (2018) proposed the gating strategy for selection of adipocyte population by applying a larger nozzle (150 μm diameter), lowering the sheath pressure to 6 psi, and enhancing the detection of the larger events in flow cytometry. However, with the limitations of in-hand technology, the nozzle and sheath pressure cannot be adjusted. Increasing of event alone could not solve the problem. Adipocyte specific markers are alternative solution for discrimination of adipocytes from pre-adipocytes. The mature 3T3-L1 adipocytes typically display phenotypic characteristics of white adipocytes (Morrison, & McGee, 2015). The amino acid transporter Asc-1 is suggested as a white adipocyte-specific cell surface protein (Ussar et al., 2014). The accumulation of intracellular lipid droplets is a distinct characteristic of mature adipocytes. These droplets can be stained by BODIPY, the fluorescent neutral lipid dye (Moden et al., 2012). Fluorescent staining of Asc-1 or lipid droplet is promising for troubleshooting.

5. Conclusion

The murine 3T3-L1 adipocytes overexpressing GLUT4-HA was successfully established in this study. However, the system for quantitating GLUT4-HA translocation by flow cytometry was not valid. Better strategy for separation of mature adipocytes from pre-adipocytes is necessary for improvement of the system.

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