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Identification of Biochemical Differences in White and Brown Adipocytes Using FTIR Spectroscopy

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Abstract: This study was conducted to investigate the developmental characteristics of adipocytes and to identify selectively white and brown adipocytes through Fourier transform infrared (FTIR) spectroscopy. For the developmental characterization of adipocytes, cells and conditioned media of white and brown adipocytes were respectively collected and analyzed. A higher amide I/amide II ratio was observed in the conditioned medium of brown adipocyte than in that of white adipocyte, indicating differences in secretory protein profiles. In contrast, an amide I/amide II ratio was higher in white adipocytes than in brown adipocytes, and mature adipocytes have higher lipid amounts than pre-adipocytes. Lipid acyl chain length was the longest in white adipocytes. These differences suggested that FTIR spectroscopy can be used to characterize developmental stages and/or types of adipocytes. To identify the possibility of selectively classifying adipose-derived stem cells, FTIR spectroscopy spectra were obtained in cells before/after white/brown adipocyte differentiation using FTIR spectroscopy and then analyzed by the principal component analysis method. All data indicated that the discrimination between adipocytes was possible in the analysis of the infrared spectroscopy spectrum by the principal component analysis technique. This study suggested the possibility of FTIR spectroscopy as a new type of cell sorting system without tagging.

Keywords: FTIR spectroscopy; adipocytes; obesity

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1. Introduction

Fourier transform infrared (FTIR) spectroscopy is a kind of microscope to view sample images using the infrared region light source and was developed in the late 1970s [1]. Unlike other optical microscopes with absorbing glass optics, it uses reflex optics to cover the entire infrared spectral region [2]. FTIR spectroscopy that is made of both a FTIR spectrometer and a microscope provides optical visualization and infrared spectral data. FTIR spectroscopy has been applied in various areas, including polymer science, forensic science, semiconductors, and (bio) chemical analyses.

Infrared spectral data, which are mostly used in chemical and physical sciences, provide information on molecular vibrations absorbing particular energy when infrared light is irradiated on a molecular bond. They were originally employed to obtain information on molecule structure, but are now used to describe any chemical changes in chemical processes. Since the infrared spectra of materials are based on molecular structures, they may be used in both qualitative and quantitative assessments to inspect raw materials in the pharmaceutical and chemical industries [3]. In the biomedical area, FTIR spectroscopy is applied to identify bacterial and fungal infection [4] and to find collagen and proteoglycan in bovine cartilage [5] as a tool for tissue diagnosis [6].

Obesity is a state in which adipocytes in the body are abnormally accumulated. Obesity generates various social, economic, and medical issues and is possibly related to the development of various human diseases such as cardiovascular disease, type II diabetes,

infertility, osteoarthritis, and certain cancers [7,8]. Obesity involves both white and brown adipocytes. White adipocytes have a unilocular lipid droplet shape, have low mitochondrial density, lack the thermogenic marker mitochondrial proton pump uncoupling protein 1 (UCP1), and have function as energy storage and endocrine regulation. Brown adipocytes contain multilocular lipid droplets, have a high mitochondrial density and UCP1, and their role is thermogenesis and endocrine regulation. Despite the severity of obesity, numerous fundamental studies have been conducted, but few of them used FTIR spectroscopy [9–13]. Thus, this study was conducted to characterize and distinguish brown and white adipocytes by using FTIR spectroscopy.

2. Materials and Methods

2.1. Culture and Differentiation

The immortalized brown preadipocyte cell line was kindly provided by Dr. Shingo Kajimura (UCSF, San Francisco, CA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 1% (*v/v*) antibiotic solution and 10% (*v/v*) fetal bovine serum (FBS) in a humidified atmosphere containing 5% (*v/v*) carbon dioxide (CO_2) (from Corning, Glendale, AZ, USA). For brown adipocyte differentiation, cells were cultured to confluence in a maintenance medium containing 10% FBS and then induced to differentiate in a differentiation medium supplemented with 0.5 mM isobutyl methylxanthine (IBMX), 0.5 µM dexamethasone, 20 nM insulin, 1 nM 3,3′,5-triiodo-L-thyronine (T3) and 125 µM indomethacin for two days (all from Sigma, Ronkonkoma, NY, USA). The cells were further induced to differentiate and mature for six days in the growth medium composed of DMEM, 10% FBS, 20 nM insulin, and 1 nM T3.

For 3T3-L1 white adipocytes, white preadipocytes were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM containing 10% (*v/v*) bovine calf serum (BCS, Corning, Glendale, AZ, USA) until confluent, and were then maintained in the same medium for an additional two days. Differentiation was induced two days post confluence by DMEM containing 10% FBS, 0.5 mM IBMX, 1 mM dexamethasone and 5 mg/mL insulin. The differentiation medium was changed to DMEM supplemented with 10% FBS and 5 mg/mL insulin every 48 h, until 10 days. Conditioned media were collected 48 h after incubation and when the brown adipocyte progenitor cells and white adipocyte progenitor cells were subcultured. The brown adipocytes were sampled six days after induction and the white adipocytes were sampled on the 8th day of induction.

2.2. Oil-Red-O Staining

Differentiated white and brown adipocytes were washed with phosphate-buffered saline (PBS, Corning, Glendale, AZ, USA) and fixed with 4% (*w/v*) paraformaldehyde (Daejung, Gyeonggi-do, Korea) for one hour at room temperature. The cells were stained with a 0.5% (*w/v*) filtered Oil-red-O (Sigma, Ronkonkoma, NY, USA) solution in isopropanol for one hour at room temperature and washed with PBS. The stained lipid droplets by Oil-red-O dye were observed under a microscope.

2.3. Reverse Transcription Quantitative Polymerase Chain Reaction Analysis

Total RNA was isolated from cultured cells using TRIzol (Invitrogen, Waltham, MA, USA). Synthesis of cDNA was carried out using 1 µg total RNA, 100 µM oligo dT primers, and a cDNA synthesis kit (Nanohelix, Daejeon, Korea). Quantitative PCR (qPCR) was performed with a StepOnePlus Real-Time PCR system (ThermoFisher, Waltham, MA, USA) using a Premier qPCR kit (Nanohelix, Daejeon, Korea) to detect specific genes for the white and brown adipocyte differentiation. Table 1 shows primers used in this study:

Table 1. List of primers (sequence 5′→3′) used for qPCR.

Target Gene	Forward Primer	Reverse Primer
Gapdh	GGGGTCCCAGCTTAGGTTCAT	TTCACACCGACCTTCACCATT
Ucp1	AGGATTGGCCTCTACGACTCA	GCATTCTGACCTTCACGACCT
Pgc1α	TCTGGGTGGATTGAAGTGGTG	TCTGTGAGAACCGCTAGCAAG
Pparγ	TCCATTCACAAGAGCTGACCC	GGCCATGAGGGAGTTAGAAGG
Prdm16	ACAAGTCCTACACGCAGTTC	CGTGTAATGGTTCTTGCCCT
aP2	GCTTTGCCACAAGGAAAGTG	ACGCCCAGTTTGAAGGAAAT
Cebpα	TGGACAAGAACAGCAACGAGT	ACCTTCTGTTGCGTCTCCAC

2.4. Preparation and Collection of Infrared Spectroscopy Spectra

Cells and media were fixed by drying on a Calcium Fluoride (CaF_2) window (Bruker Optics, Leipzig, Germany). The cells used precursor and mature cells in both white and brown adipocytes. The conditioned medium was also measured before and after induction of differentiation. FTIR spectra were collected by LOMUS (Bruker Optics, Leipzig, Germany) with 2 cm^{-1} resolution, 5 × 5 μm, and 16 scans per pixel, pre-processed (removing CO_2 and H_2O peaks using 'atmospheric compensation' function and Rubberband baseline correction), compared by "maximize each spectrum' function in specific wavenumber regions, and PCA was analyzed by OPUS 7.7.42 (Bruker Optics, Leipzig, Germany).

2.5. Statistical Analysis

All data were analysed using the Student *t*-test and/or one-way ANOVA followed by a post hoc test for multiple comparisons using GraphPad Prism 6 (USA). Values with $p < 0.05$ were considered as significant.

3. Results

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3.1. Differentiation of 3T3-L1 White Preadipocytes and Immortalized Brown Preadipocytes

Differentiation into white adipocytes and brown adipocytes was induced, and the differentiated white adipocytes and brown adipocytes were identified by staining fat droplets with Oil-Red-O (Figure 1A,B). In addition, lipid accumulation during the differentiation of brown adipose progenitor cells was confirmed (Figure 1A,B). To confirm the differentiation from immature adipocytes to mature adipocytes, the expression of specific transcriptional regulators was analyzed (Figure 1). Peroxisome proliferator-activated receptor gamma (PPARγ), adipocyte protein 2 (aP2), and CCAAT/enhancer-binding protein alpha (C/EBPα), which are specific transcription factors for white adipocytes, were identified (Figure 1A). In addition, PR domains containing 16 (PRDM16), PPARγ, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α), and uncoupling protein 1 (UCP1), which are specific transcription factors for brown adipocytes, were identified (Figure 1B).

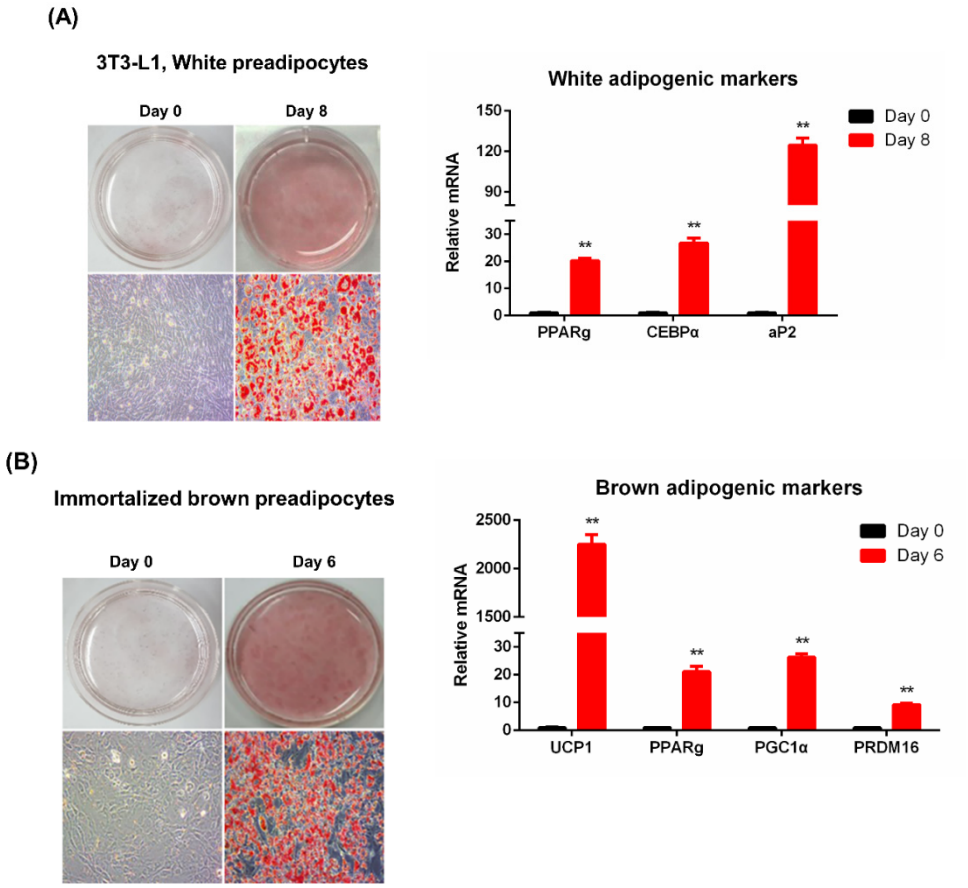

Figure 1. Characteristics of adipocytes assessed by Oil-Red-O and expression of adipocyte markers. (**A**) Expression of white adipocyte markers in white adipogenesis (white adipocyte differentiation) using RT-PCR analysis. (**B**) Expression of brown adipocyte markers in brown adipogenesis (brown adipocyte differentiation) using RT-PCR analysis. MWCM: mature white adipocyte conditioned medium; WPCM: white preadipocyte conditioned medium; WAM: white adipocyte medium; MBCM: mature brown adipocyte conditioned medium; BPCM: brown preadipocyte conditioned medium; BAM: brown adipocyte medium. ** $p < 0.01$.

3.2. FTIR Spectroscopy Spectra of Adipocyte Conditioned Media

Comparing infrared spectra of mature white adipocyte conditioned medium (MWCM) (red line), white preadipocyte conditioned medium (WPCM) (green line), and white adipocyte medium (WAM) (blue line), MWCM showed a different spectrum from WPCM and WAM (Figure 2A). Low amide I (1652 cm^{-1})/II (1543 cm^{-1}) bands and a unique ring C-C stretch of phenyl (1590 cm^{-1}) were detected in the spectrum of MWCM, but a high amide I/II and no peak related to ring C-C stretch of phenyl were detected in the spectra of WPCM and WAM (Figure 2A). In addition, a ring base (1559 cm^{-1}) band was not found in the spectrum of MWCM (Figure 2A). On the other hand, comparing infrared spectra of mature brown adipocyte conditioned medium (MBCM) (pink line), brown pre-adipocyte conditioned medium (BPCM) (green line), and brown adipocyte medium (BAM) (blue line), BPCM showed a different spectrum from MBCM and BAM. However, BPCM did not show absorbance band at 1590 cm^{-1} (Figure 2B) corresponding to C=N, NH2, adenine molecular (1589 cm^{-1}), or ring C-C stretch of phenyl (1590 cm^{-1}). In the spectra of MWCM and MBCM, both did not show a 1559 cm^{-1} peak but a 1590 cm^{-1} peak (Figure 2A). Regarding the amide I/II ratio, WAM (1.12), WPCM (1.29), and MWCM (1.34) were similar, whereas a difference was detected among BAM (1.46), BPCM (3.96), and MBCM (2.42) (Figure 3A). Protein amount (amide I/I + II) showed WAM (0.53), WPCM (0.56), MWCM (0.57) BAM (0.59), BPCM (0.79) and MBCM (0.71), respectively (Figure 3B).

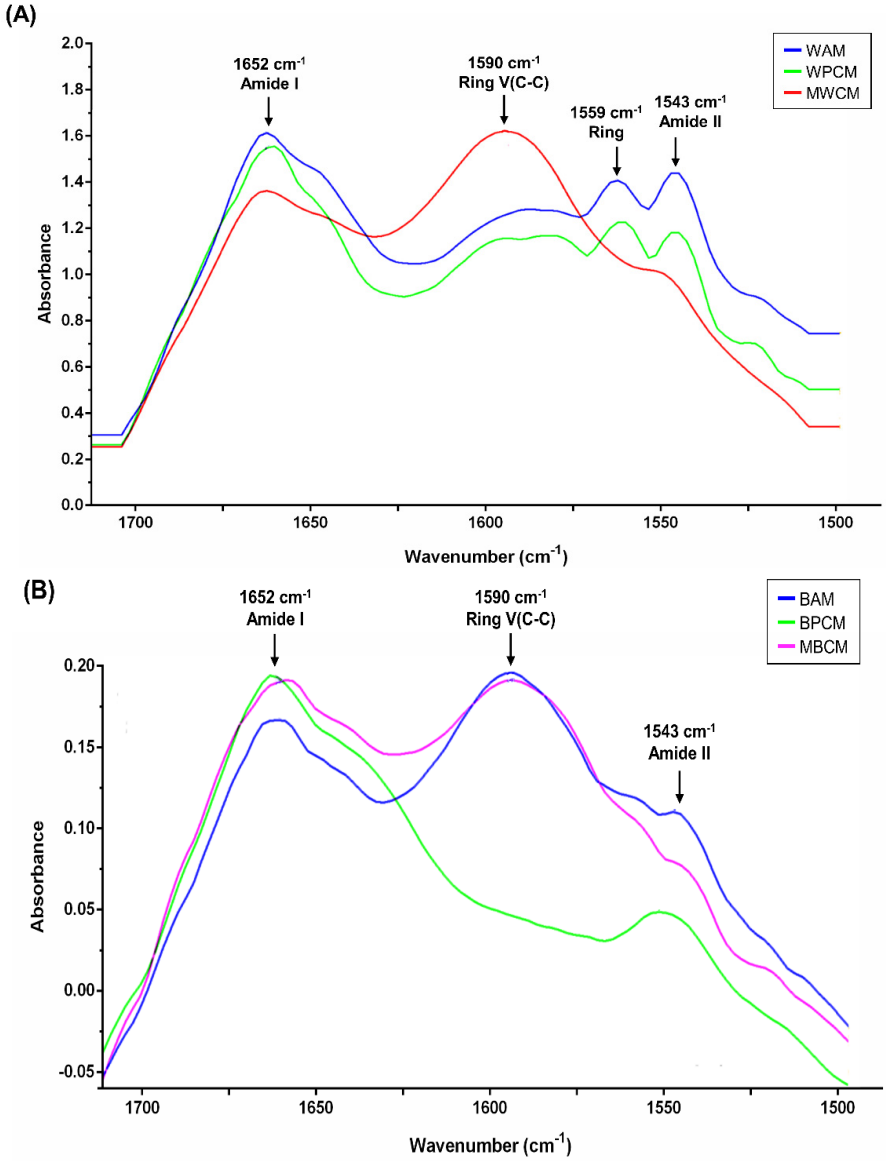

Figure 2. FTIR spectra of various adipocyte-conditioned media. (**A**) MWCM (red), WPCM (green) and WAM (blue), (**B**) MBCM (pink), BPCM (green) and BAM (blue).

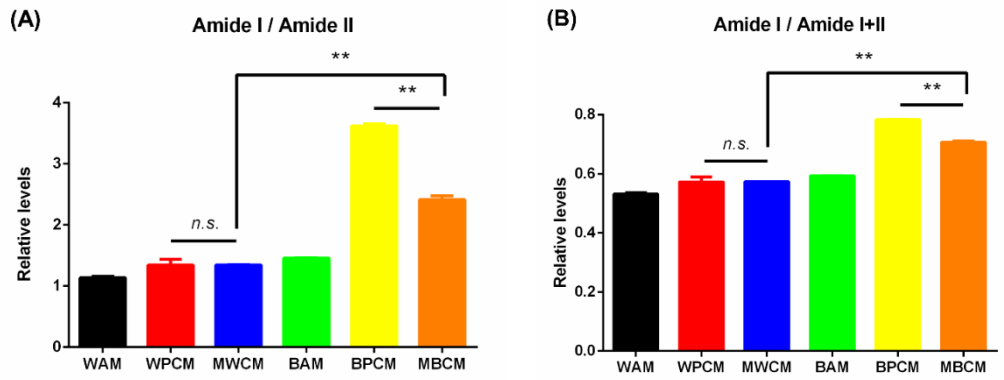

Figure 3. The difference of (**A**) amide I/amide II and (**B**) amide I/amide I + amide II of various adipocyte conditioned media. WAT: white adipocytes; WPA: white preadipocytes; BAT: brown adipocytes; BPA: brown preadipocytes. ** $p < 0.01$; *n.s.*, not significance.

3.3. FTIR Spectroscopy Spectra of Adipocytes

Comparing infrared spectra of white preadipocytes (WPA) (red line) and white adipocytes (WAT) (pink line), WAT showed an acyl chain of CH_2 asymmetric (asym)/symmetric (sym) stretching vibration (2922/2852 cm^{-1}) strongly and an acyl chain CH2 bending (1469 cm^{-1}) weakly (Figure 4A). On the other hand, comparing infrared spectra of brown preadipocytes (BPA) (green line) and brown adipocytes (BAT) (blue line), BAT showed a clear CH_2 symmetric stretching (2852 cm^{-1}), an acyl chain of CH2 scissoring mode (1465 cm^{-1}, related to lipid) and a peak (1444 cm^{-1}, related lipid, and fatty acid) strongly (Figure 4B), but a weak amide II band was observed at 1540 cm^{-1}. Lipid (CH_2 sym/asym) was observed most in WAT and BAT (Figure 5A). Amide I/II ratios related to protein were higher in WAT and WPA than in BAT and BPA (Figure 5B). Moreover, the lipid acyl chain length (CH_2 asym/CH_3 asym) was highest in WAT (Figure 5C).

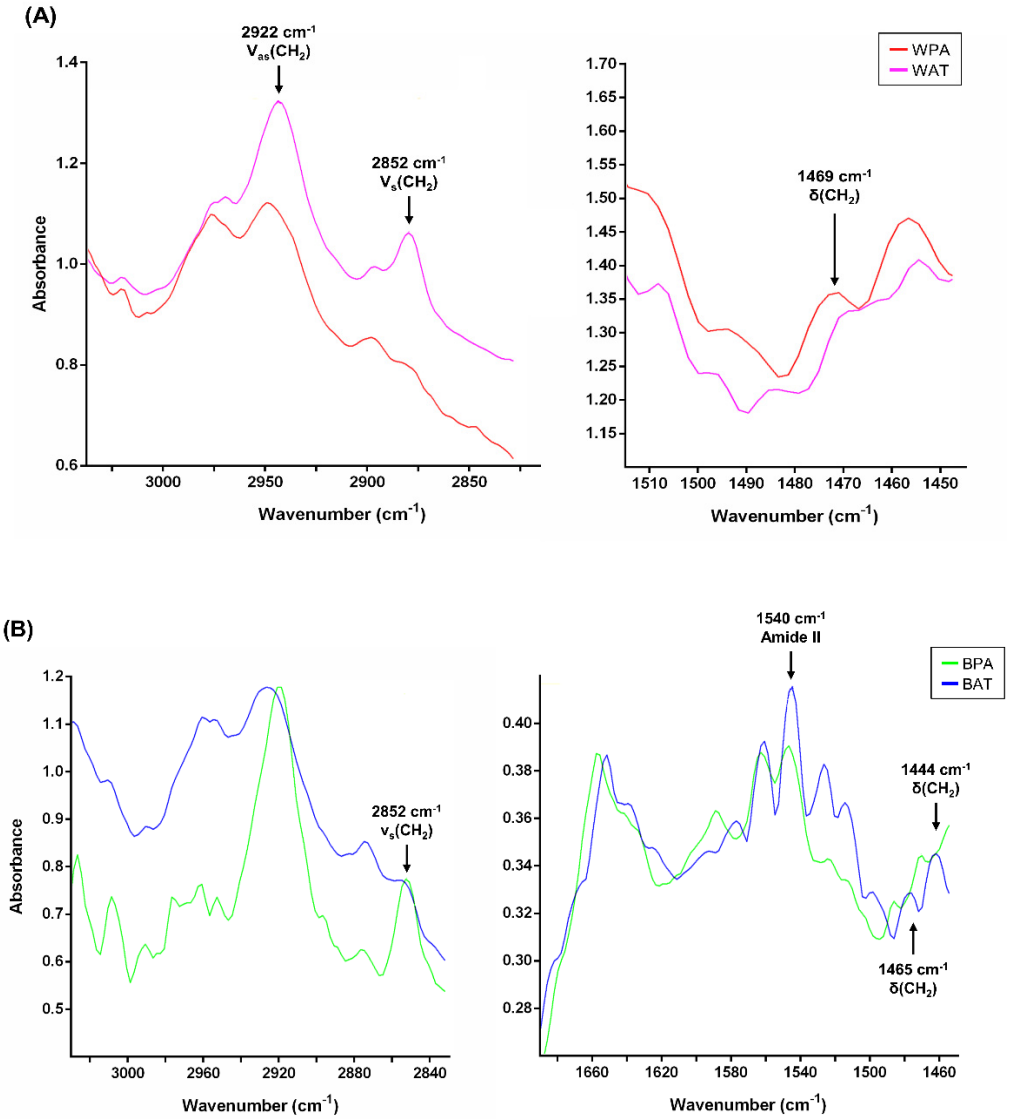

Figure 4. FTIR spectra from different types of adipocytes. (**A**) WPA (red), WAT (pink), (**B**) BPA (green), BAT (blue).

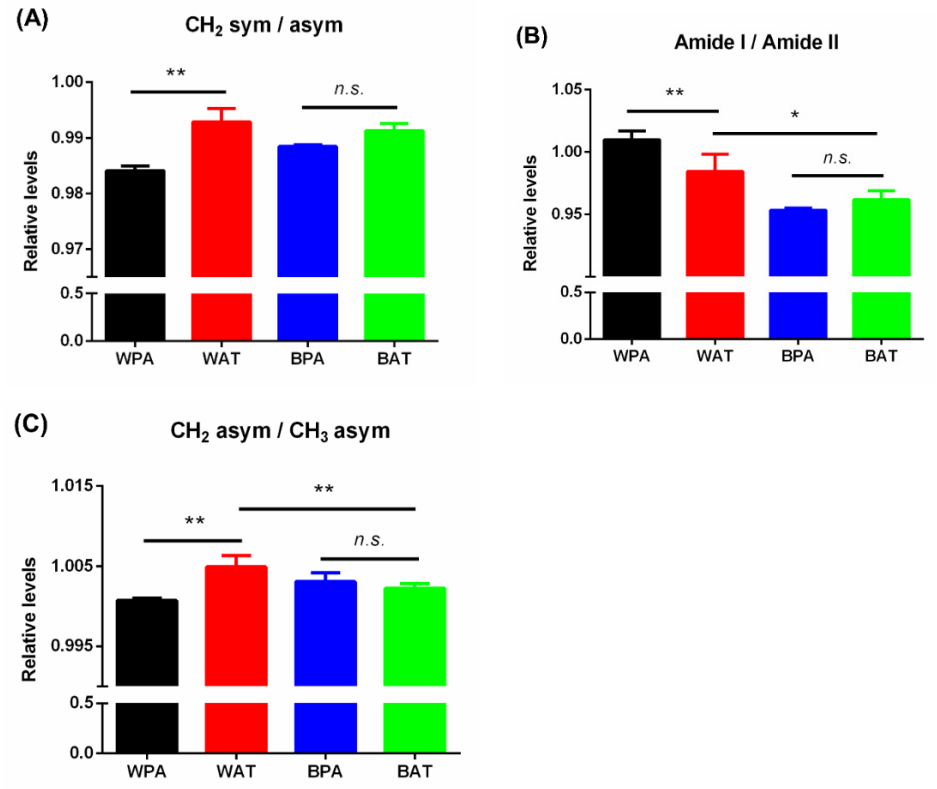

Figure 5. Analyses of FTIR spectra on different types of adipocytes—(**A**) CH_2 sym/asym, (**B**) amide I/II ratio, and (**C**) CH_2 asym/CH_3 asym. Sym: symmetric stretching; asym: asymmetric stretching. * $p < 0.05$; ** $p < 0.01$; *n.s.*, not significance.

3.4. Discrimination of Adipocytes

To further confirm that the expression of differentiation could be discriminated by FTIR, various adipocytes were collected and subjected to FTIR analysis. In the FTIR spectra when raw infrared spectra were converted into first derivation, seven smoothing points, and vector normalization conditions, the difference was found in the 3000~2840 and 1740~1340 cm^{-1} regions. Unique FTIR spectra were also found in adipogenic differentiation. FTIR spectra showed the difference at two important regions, such as the lipid region (3000–2830 cm^{-1}: CH stretching), (1750–1700 cm^{-1}: C=O ester lipid), (1450–1330 cm^{-1}: CH bending), and the protein region (1700–1500 cm^{-1}: amide I and amide II protein). Additionally, four groups of spectra were separated into 2D PCA sore and 3D PCA score plots (Figure 6A,B).

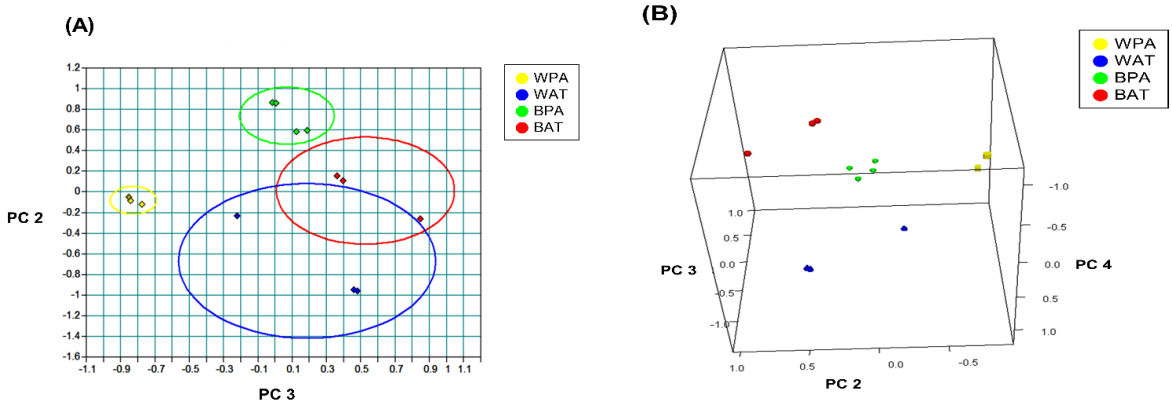

Figure 6. Discrimination of adipocytes by principal component analysis. (**A**) Two-dimensional drawing of identification by PCA, (**B**) three-dimensional drawing of identification by PCA.

4. Discussion

Obesity is a state in which adipocytes in the body are abnormally accumulated. Obesity generates various social, economic, and medical issues, and is possibly related to the development of various human diseases, such as cardiovascular disease, type II diabetes, infertility, osteoarthritis, and certain cancers [7,8]. Recently, concerning the problem of obesity, research on brown fat cells or browning has been actively conducted [14,15]. Brown fat cells are cells that consume a lot of energy as heat energy due to non-shivering thermogenesis mediated by UCP1 of a large number of mitochondria. In terms of brown fat cells, a previous study discussed the involvement of pannexin-1 channels in adaptive thermogenesis in brown adipose tissue [16]. In addition, flap necrosis in reconstructive surgery is a common surgical complication, and to treat this, adipose-derived stem cells are used. Like this report, the use of adipose-derived stem cells is increasing [17]. If a promising mechanism of browning is discovered in the future, it will cause a drastic change in the treatment of obesity. Thus, this study investigated whether the FTIR system can be applied for characterizing adipose cells.

The differentiation of adipocytes for this study was confirmed through Oil-red-O staining and the presence of the following markers: white adipocyte markers (PPARγ, C/EBPα, aP2) and brown adipocyte markers (PRDM16, PPARγ, PGC-1α, UCP1). PPARγ is a key regulator of adipocyte differentiation and is an important transcription factor for adipocyte metabolism and differentiation [14]. C/EBPα directly leads to lots of adipocyte genes [14]. UCP1 is expressed distinctly in brown adipocytes as heat without generating ATP. PGC-1α is a marker that leads to mitochondrial biogenesis and UCP1 expression [18]. PRDM16 is a marker to control the fate of brown adipocytes [19]. Since Oil-red-O staining and the above specific markers confirmed the identity of adipocytes (Figure 1A,B), these adipocytes were used as a sample for the next experiment.

In FTIR comparison with conditioned media of adipocytes (Figure 2A,B and Supplementary Figure S1A,B), amide I/amide II ratios were different between MBCM (2.42) and BPCM (3.96) (Figure 3A) [20,21]. It is believed that high amide I/amide II ratios affect protein pattern composition [22]. BPCM had the highest protein content (0.79) (Figure 3B) [20,21]. Therefore, the differentiation process of brown adipocytes is presumed that the function of secretory protein affects differentiation differently from that of white adipocyte differentiation. In FTIR comparison with adipocytes (Figure 4A,B and Supplementary Figure S1C,D), various chemical constituents such as lipid amount, amide I/amide II ratio, and lipid acyl chain length in adipocyte types were analyzed to determine any difference [20,21]. It was confirmed that differentiated adipocytes contained more fat mass than progenitor cells (Figure 5A) [20,21]. Unlike in conditioned media, amide I/amide II ratios were similar in the cells, showing slightly higher from WPA and WAT (Figure 5B) [20,21]. Because amide absorption is sensitive to protein formation, the amide I/amide I + amide II ratio is an indicator of protein level [20]. Protein rate is an important aspect of cell regulation during differentiation [23,24]. Therefore, there is a less significant change in the composition and content of intracellular proteins, but it can be deduced that small differences affect the differences among cells, especially in the differentiation of white adipocytes. WAT showed the longest lipid acyl chain lengths (Figure 5C) [20,21]. Lipid acyl chain length has important effects on cholesterol orientation in monounsaturated bilayers [25]. This phenomenon was inferred to the functional difference between WAT and BAT. This is the first study to examine the properties of adipocyte-conditioned medium and adipocytes at different stages of development using FTIR spectroscopy.

Based on the findings obtained in this study, it is expected that when a cell-conditioned medium without preparation is analyzed by an infrared microscope with high resolution, it will be possible to perform metabolic studies, structural analysis, and protein changes such as alpha-helix and beta-sheet of proteins released in the differentiation process of adipocytes [26]. Various adipokines, for example, have been discovered through the investigation of variations in protein secretion during adipocyte development and a difference in the secreted proteins was noted during the differentiation process. If infrared analysis with

high resolution would be applied, it would be possible to expand the research of secretory proteins [27]. When obesity-related diseases are recognized as global issues, attention is focused on beige adipocytes. The differences in the expression of the Hox family were raised in this process [28–31]. In studies, when a deep understanding of the cell is needed, the application of infrared microscope with high resolution is required to provide cell-related chemical information by the non-invasive method. Therefore, this paper presented the future direction of adipocyte characterization studies using advanced FTIR spectroscopy as above and is expected to contribute information in research using FTIR when searching for obesity-related important metabolites and anti-obesity drug candidates.

Furthermore, cell differences between 3000 and 2840 and between 1740 and 1340 cm^{-1} of the pre-treated spectra were observed (Supplementary Figure S2). FTIR spectra showed the difference of WPA, WAT, BPA, and BAT at two important regions, both the lipid region and the protein region. In this regard, further research through molecular biological analysis in connection with FTIR is likely to be needed. These spectral differences enabled the discrimination of adipocytes when applying principal component analysis. It was found that mature adipocytes were widely distributed, which was presumably due to cell differentiation (Figure 6A,B). Additionally, cluster by dendrogram was tested but was less than PCA (Supplementary Figure S3). Currently, there is a growing need for research on adipose-derived stem cells, whereby a method for selectively screening only adipose-derived stem cells from adipose tissue is necessary. FACS, which is currently used as a cell sorter, basically requires tagging of cells. However, the cell labelling process is not necessary for an infrared microscope system, making it possible to develop an inexpensive and efficient device in connection with a kind of microfluidic cell sorter. In this way, we presented a brand-new cell sorter using FTIR spectroscopy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12063071/s1, Figure S1: Raw spectra of conditioned media [WAM (black), WPCM (red), MWCM (blue), BAM (green), BPCM (yellow), MBCM (orange)] and adipocytes [WPA (black), WAT (red), BPA (blue), BAT (green)]. (A) Full-range scale spectra of adipocyte conditioned media (B) Adipocyte conditioned media' spectra between 1850 cm^{-1} and 1340 cm^{-1} (C) Full-range scale spectra of adipocytes (D) Adipocytes' spectra between 1850 cm^{-1} and 1360 cm^{-1} marked with positions of 1640 cm^{-1} (peak suspected by the effect of water) and 1634 cm^{-1} (water peak), Figure S2: Spectra used in PCA for discrimination of adipocytes, Figure S3: Cluster by dendrogram for discrimination of adipocytes.

Author Contributions: Conceptualization, D.S. and S.P.; methodology, D.S. and S.P.; software, D.S.; validation, D.S., S.P., S.Y. and Y.K.; formal analysis, D.S. and S.P.; investigation, D.S. and S.P.; resources, D.S. and S.P.; data curation, D.S. and S.P.; writing—original draft preparation, D.S. and S.P.; writing—review and editing, Y.K.; visualization, D.S. and S.P.; supervision, Y.K.; project administration, D.S. and S.P.; funding acquisition, D.S., S.P. and Y.K. All authors have read and agreed to the published version of the manuscript.

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