A Thesis
For the Degree of Master of Science

Studies on gene expression pattern in subcutaneous and visceral adipose tissue derived stromal cells

피하 및 내장지방 유래 기질세포에서의 유전자 발현 양상 연구

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SUMMARY

Adipose tissue is divided into two types such as visceral and subcutaneous adipose tissue. Visceral and subcutaneous adipose tissues have different properties and risk of diseases. Visceral obesity is known to be more closely related to metabolic disorder than peripheral obesity. Several studies were performed to analyze gene expression of mature adipocytes and adipocyte precursors from subcutaneous and visceral fat.

First, adipose-derives stromal cells (ADSC) were isolated using MACS separation. $CD31^-/CD45^-$ cells were selected, plated to investigate gene expression of ADSC at passage 2. Gene expression analysis of ADSC from subcutaneous and visceral depots of human was revealed depot-specific differences in expression of multiple genes in 810 of 20,313 genes ($P < 0.05$). Significantly expressed genes were involved various different gene ontology.

Among differentially expressed genes, we focused on five energy-metabolism related genes of S-ADSC and V-ADSC. In V-ADSCs, TCF21 and TM4SF1 were overexpressed, whereas MMP3 was higher in S-ADSC than in V-ADSC.

TCF21 and TM4SF1 that highly expressed in V-ADSC were associated with dysregulated lipid metabolism, however MMP3 that overexpressed in S-ADSC have some relationship with protective effect in obesity.

We evaluated the characteristics of ADSC isolated from subcutaneous (S-ADSC) and visceral (V-ADSC) fat depots by FACS using CD31, CD45, CD34, CD73, CD90 and CD105 antibodies, and assessed the differentiating potentials of ADSC into adipocyte, osteocyte and chondrocyte. Immunophenotyping showed that MACS separation and plastic culturing selects homogeneous cell populations of ADSC, sharing typical markers of mesenchymal stem cells. Similar to S-ADSC, V-ADSC differentiated in vitro toward adipogenic, osteogenic and chondrogenic lineages, suggesting partially the multipotency of such stem cells. However, S-ADSC had higher proliferation and differentiation potential into adipocytes than V-ADSC.

These results indicate that S-ADSC and V-ADSC show distinct patterns of gene expression and may explain intrinsic differences of subcutaneous and visceral fat. Besides, considering expression of energy-metabolism related genes, adipocytes from subcutaneous and visceral depots could be fundamentally the different in their properties. These

differences of adipose-derived stromal cells might be helpful to understand molecular mechanism. Furthermore, it could be present therapeutic targets for metabolic disorders.

Keywords : human, adipose-derived stromal cell, subcutaneous, visceral, gene expression, microarray, energy metabolism.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADSC : adipose-derived stromal cell

BMI : body mass index

BMP : bone morphogenic protein

DEG : differentially expressed genes

DMEM : dulbecco's modified eagle's medium

FACS : fluorescence activated cell sorter

FBS : fetal bovine serum

FFA : free fatty acid

GO : gene ontology

MACS : magnetic activated cell sorter

MSC : mesenchymal stem cell

PBS : phosphate buffer saline

PCR : polymerase chain reaction

RBC : red blood cell

SAT : subcutaneous adipose tissue

SVF : stromal vascular fraction

TG : triglyceride

TGF : transforming growth factor

VAT : visceral adipose tissue

CHAPTER 1.

GENERAL INTRODUCTION

AND

LITERATURE REVIEW

1. General introduction

Obesity is a worldwide health problem that impacts the risk and prognosis of many diseases. Epidemiological studies have found that there are two different types of obesity, such as peripheral and visceral obesity. Of these, visceral obesity determined by high waist–hip ratio, has been reported to be associated with metabolic disorders, including insulin resistance, type 2 diabetes mellitus and cardiovascular disease (Despres et al., 1990; Carey et al., 1997). Consistent with these findings, removal of visceral fat by omentectomy resulted in decreased levels of insulin and glucose (Thorne et al., 2002). In contrast, removal of subcutaneous fat by liposuction did not improve any aspect of metabolic syndrome (Klein et al., 2004).

Body fat is composed of two functionally different lineages of adipose tissue: brown and white adipose tissue. Of these, white adipose tissue comprises the majority of the body fat, and is responsible for energy storage. White adipose tissue is also subdivided into two types according to its locations, such as visceral and subcutaneous depots. There have been several studies suggesting that visceral and subcutaneous adipocytes

behave differently in both morphologic and metabolic aspects. Visceral adipocytes tend to be larger in size and more insulin-resistant (Frayn, 2000; Dicker et al., 2009). In addition, visceral adipocytes have a greater lipolytic activity than subcutaneous adipocytes. Two adipocytes have differential gene expression suggesting these differences may contribute to functional properties of adipocytes (Vidal, 2001; Atzmon et al., 2002).

Furthermore, preadipocytes, precursors of adipocytes, from different depots in the same individuals have been reported to have distinct characteristics, including capacities of replication, differentiation and apoptosis (Tchkonia et al., 2005; Tchkonia et al., 2006). Recent studies have reported that the two types of adipocytes are also different in gene expression profiles. Studies using adipocytes or preadipocytes demonstrated that expression profiles of developmental genes are different between the visceral and subcutaneous fat (Gesta et al., 2006; Tchkonia et al., 2007), suggesting genetically programmed developmental differences between the two separate adipose tissues from different regions.

Adipose tissue-derived stromal cells (ADSC) are multipotent precursor cells from adipose stroma which have a

potential to differentiate into various cell types. Given that mature adipocytes are post-mitotic, the ability to expand adipose tissue and to maintain adipose dynamics depends on the presence of proliferative adipocyte precursor cells. ADSC are considered to reside in adipose stromal vascular fraction (SVF) (Rodeheffer et al., 2008) and to be responsible for generation of adipocytes and preadipocytes (Otto and Lane, 2005).

Similar to adipocytes, ADSC from two separate depots have been reported to exhibit different characteristics. Comparison studies demonstrated that adipose-derived stromal cells from subcutaneous fat (S-ADSC) are more adipogenic and osteogenic than adipose-derived stromal cells from visceral fat (V-ADSC) (Toyoda et al., 2009). In addition, insulin is more lipogenic and less mitogenic in V-ADSC compared to S-ADSC (Sadie-Van Gijsen et al., 2010). However, there has been no study evaluating the differential expression levels of genes between V-ADSC and S-ADSC.

Adipose-derived stromal cells possess the ability to differentiate into multiple lineages, a property that might be of value for the repair or replacement of various damaged cell types (Gimble et al., 2007; Schaffler and Buchler, 2007). Adipose tissue transplantation has primarily been used as a tool

to study physiology and for human reconstructive surgery. However, transplantation of ADSC is now being explored as a possible tool to promote the beneficial metabolic effects of subcutaneous white adipose tissue and brown adipose tissue, as well as adipose tissue. To be clear the characteristics of ADSC from two depots, it is necessary to understand their genetic part list at the levels of mRNA and regulatory network. Advances in genomic technologies have allowed capture of genetic information at each expression levels of ADSC and have contributed enormously to understanding of the fundamental processes.

In this study, we evaluated the gene expression profiles of ADSC from subcutaneous and visceral depots to explore the hypothesis that ADSC from distinct anatomic locations have intrinsic difference. To identify genes being upregulated in S-ADSC and V-ADSC, we used a microarray technology and a gene network map. Gene ontology annotation was carried out for assuming differences in cellular function.

2. Literature review

1) Adipose tissue

Fat is existent in various anatomical positions which collectively called "the adipose organ". Adipose tissue regulates energy balance because it is not only a lipid storing and mobilizing tissue but also functionally specialized tissues to produce heat and to produce or release a number of cytokines. In addition to role for energy storage, adipose tissue is important for physiological functions and metabolism. However, excess accumulation of body fat is also harmful to health, as obesity is strongly associated with development of diabetes, hypertension and heart disease. It is clear that too little and too much fat can cause or worsen serious health problem.

In general, the adipose organ is divided into two functionally different tissues : white adipose tissue and brown adipose tissue, whose main functions are energy storage and energy expenditure, respectively. However, white adipose tissue also shows a distinct metabolic heterogeneity in terms of visceral versus subcutaneous depot.

(1) Subcutaneous and visceral adipose tissue

The main cellular components of adipose tissue are adipocytes. The adipocytes response to insulin and other hormones in lipolytic activity differ between subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). Inflammatory cells are more common in visceral compared with subcutaneous fat (Bruun et al., 2005). Subcutaneous fat accumulation represents the normal physiological buffer for excess energy intake with limited energy expenditure. It acts as a metabolic sink where excess free fatty acids (FFAs) and glycerol are stored as triglycerides (TGs) in adipocytes (Freedland, 2004). Because of anatomical localization, VAT venous blood is flowed directly to the liver through the portal vein. However, venous drainage of SAT is through systemic veins. FFAs and adipokines secreted by visceral adipocytes can directly access to portal drainage of visceral fat.

Growth aspect (cell hyperplasia and cell hypertrophy) of the various adipose tissue regions may be linked to expression of regulating factors. The total number of adipocytes is accompanied with increasing fat mass, but increased number and proportion of large adipocytes, compared

smaller ones, may partially give failure of adipose tissue to function properly (Rajala and Scherer, 2003). The smaller adipocytes become more insulin sensitive, whereas large adipocytes tend to be insulin resistant and contribute more to the metabolic problems associated with obesity. There was report that preadipocytes from the SAT have a greater differentiation potential than those from the VAT (Hauner et al., 1988). This increases the number of smaller adipocytes in SAT with a high tendency for FFAs and TG uptake. Because of this differences, SAT and VAT might show to be distinct risk of disease.

(2) Regional differences in metabolism

Noticeable regional differences of metabolism in white adipose tissue have been reported. Epidemiological studies have found that increased visceral adipose tissue is associated with adverse health risk, such as insulin resistance, type 2 diabetes mellitus, dyslipidemia, hypertension and overall mortality (Carey et al., 1997; Wang et al., 2005; Zhang et al., 2008). By contrast, peripheral obesity seems to be associated with improved insulin sensitivity and a lower risk of type 2

diabetes mellitus than individuals with central obesity (Misra et al., 1997; Snijder et al., 2003). These different metabolic activities and their vascular connections are believed to be crucial factors in predisposing for obesity complications (Franco et al., 2001; Lemieux, 2001). As an example, mesenteric fat cells have excessive rate of glucose metabolism and lactate production than those of fat cells from other adipose depots. Although the basal lipolytic rate of mesenteric fat cells is higher than that of adipocytes from other regions, the response to lipolytic hormone is decreased (Storck and Spitzer, 1974).

(3) Regional differences in gene expression

For genome analysis of adipose tissue and progenitor, microarrays have been used to profile gene expression change that related with the state of differentiation to compare visceral with subcutaneous fat (Linder et al., 2004; Wolfs et al., 2010). The significant differences in gene expression and secretion of adipose produced factors demonstrate the metabolic differences between the two adipose tissue types (Wajchenberg, 2000; Stears and Byrne, 2001; Vidal, 2001; Atzmon et al., 2002;

Wajchenberg et al., 2002). In a recent study, different expression levels in subcutaneous and visceral fat are identified by DNA analysis. 150-fold differences of expressed genes were found in about 18% of all adipose tissue, confirming the biological diversity of these adipose depots (Atzmon et al., 2002).

Changes in gene expression during differentiation have been studied by comparison of differentiated 3T3-L1 adipocytes and nondifferentiated 3T3-L1 preadipocytes (Guo and Liao, 2000). A vast difference in expression patterns was discovered. From this analysis, it could be generated that the molecular signatures of adipose tissue that define the individual components and the pathways that regulate adipocytes differentiation.

2) Adipose derived stromal cells

(1) Adipose-derived stromal/stem cells

Mesenchymal stromal cells (MSCs) can routinely be isolated from several organs, such as fetal liver, umbilical cord

blood, bone marrow and adipose tissue (Wagner et al., 2005; Kern et al., 2006).

The adipose tissue is highly complex tissue and consists of mature adipocytes, preadipocytes, fibroblasts, smooth muscle cells, endothelial cells, monocytes/macrophages (Weisberg et al., 2003), and lymphocytes (Caspar-Bauguil et al., 2005). The stromal-vascular fraction (SVF) of the adipose tissue has been focused on stem cell research (Prunet-Marcassus et al., 2006), since this tissue compartment provides a rich source (Zuk et al., 2002) of pluripotent adipose tissue-derived stromal cells. There is discrepancy using terms describing multipotent precursor cells from adipose tissue stroma, such as adipocyte precursor cells (Dudas et al., 2008), preadipocytes (Taniguchi et al., 2008), adipose stroma vascular cell fraction, adipose tissue-derived stromal/stem cells (ADSC) (Liu et al., 2009) and others. The plastic adherent cell stromal population by collagenase dissociation of adipose tissue have been termed "Adipose-derived Stem Cells" (ASCs) based on a consensus by the Second Annual Meeting of the International Fat Applied Technology Society (Pittsburgh, PA, 2004).

Because of heterogeneous adipose SVF fraction, there

are a few specific markers for ASCs. ASC share many cell surface markers. Markers in common with BM-MSCs include: CD13, CD29 (β1 integrin), CD44, CD58, CD90 (Thy-1), CD105 (endoglin), and CD166. Co-expression of mesenchymal, pericyte, and smooth muscle surface markers by ASCs has been shown to be greater than 90% (Traktuev et al., 2008). ASCs have been observed to alter their expression of cell surface proteins based on cell density and the number of passage and contact with tissue culture plastic (Nakagami et al., 2006). Cells in the SVF and early passaged ASCs have relatively higher expression of CD117, HLA-DR and stem cell associated markers, such as CD34, and low levels of stromal cell markers such as CD13, CD29, CD44, CD63, CD73, CD90, CD166 and CD105 (Mitchell et al., 2006; Varma et al., 2007). In general, as ASCs are passaged, expression levels of the stem cell associated marker CD34 falls, while levels of the above mentioned stromal surface markers rise. The implication of a loss in CD34 expression remain unclear, but some data indicate that $CD34^+$ ASCs are more proliferative, while $CD34^-$ ASCs possess greater plasticity (Suga et al., 2009).

ASCs display multilineage plasticity in that they able to differentiate along multiple cell lineages (mesoderm, endoderm

and ectoderm). Furthermore, ASCs possess great utility and have been shown to differentiate along many lineages, including bone, adipose, endothelial, skeletal muscle, smooth muscle, cartilage, nervous, and cardiac tissue. There is emerging evidence that ASC plasticity is donor-dependent, specifically that gender, anatomic location, and age influence differentiation potential (Ogawa et al., 2004; Aksu et al., 2008).

(2) Isolation of adipose-derived stromal cell

Histologic and electron microscopic studies identified presumable adipocyte progenitor cells within embryonic and adult adipose tissues. A recent study confirmed to continuously turn over the adipocytes in adult human (Spalding et al., 2008). Since mature adipocytes are postmitotic, adipocyte hyperplasia in adults requires that new adipocytes be produced from the differentiation of precursor cells. These findings strongly suggest that there is an adipose tissue precursor cell population resident in adipose tissue.

In the 1960's, Rodbell and colleagues (Rodbell, 1966a, 1966b) developed initial in vitro isolation methods for mature adipocytes and progenitors from rat epididymal fat. They

minced rat adipose tissue, washed to remove contaminating hematopoietic cells, incubated the tissue with collagenase and centrifuged the digest. The floating population of mature adipocytes was separated from the pelleted stromal vascular fraction (SVF). The SVF cells were plated to culture dish to select the plastic adherent population, which enriched for the preadipocytes. Approximately 1:30 SVF cells bind to the plastic surface and these can be culture expanded using growth medium (Mitchell et al., 2006). A single milliliter of human subcutaneous adipose tissue lipoaspirate yield between 250,000 to 375,000 adherent ADSC within a 4 to 7 day period (Yu et al., 2010).

(3) Subcutaneous and visceral adipose-derived stem/stromal cells

Adipokines are not only secreted by adipocytes but also by SVF cells (Fain et al., 2004), yet differences be present in the type and amount of molecules produced by each component. Accordingly, whereas adipokines such as leptin and adiponectin are primarily secreted by adipocytes (Fain et al., 2004), SVF cells contribute to most of the release of inflammatory

mediators and interleukins such as tumor necrosis factor α or interleukin 6 (Zeyda et al., 2007). Subcutaneous preadipocytes had the higher capacity to differentiate, indicating by lipid accumulation and expression of the fat cell fatty acid binding protein, FABP-4, as well as PPAR-γ and C/EBP-α. Transfection with C/EBP- α contribute to regional differences in fat depots. Differences in preadipocyte replication and differentiation remained even in colonies derived from single primary subcutaneous compared to visceral preadipocytes (Tchkonia et al., 2006; Tchkonia et al., 2007). Depot-dependent differences in preadipocyte replication, differentiation and TNF-α-induced apoptosis and developmental gene expression profiles remained remarkably similar in primary preadipocyte cultures and in preadipocyte strains created by expressing human telomere reverse transcriptase in clones derived from single human abdominal subcutaneous and visceral preadipocytes after 40 population doublings (Tchkonia et al., 2006; Tchkonia et al., 2007).These findings indicate that the distinct properties of fat cell progenitors from different fat depots are inherent.

CHAPTER 2.

Difference of gene expression in subcutaneous and visceral adipose-derived stromal cells

1. Introduction

Adipose tissue is an essential endocrine organ. Studies on adipose tissues have been attracting increasing attention because it plays opposite roles. Peripheral obesity has little or no risk of the common complications of obesity, whereas central obesity tends to present these complications (Gillum, 1987; Mauriege et al., 1993; Kissebah and Krakower, 1994; Abate and Garg, 1995). Several physiological studies have shown that two fat depots have adverse effects: removal of subcutaneous fat or its transplantation to the visceral cavity improves the metabolic criteria or no aspect, whereas removal of visceral fat decreases the insulin and glucose levels (Thorne et al., 2002; Klein et al., 2004; Tran et al., 2008).

Insights on differences in adipose depots at a molecular level have recently emerged through studies of gene expression (Cantile et al., 2003; Vohl et al., 2004). Especially, adipocytes from subcutaneous and visceral fats have revealed depot-specific differences in the expression of genes involved in embryonic development and pattern specification (Gesta et al., 2006; Tchkonia et al., 2007) which suggest genetically

programmed developmental differences between the two separate adipose tissues from different regions. Furthermore, the expression of genes that regulate development, which include homeotic genes, also differs between preadipocytes isolated from subcutaneous and visceral depots (Yamamoto et al., 2010).

By comparing the gene expression patterns of adipose-derived stromal cells (ADSCs) from subcutaneous and visceral depots, a potentially important role of genes in the control of adipose tissue distribution and function has been identified. In this chapter, the gene expression of ADSCs is described using a micro-array. Gene clustering and gene ontology annotation were used to identify genes that are being upregulated.

2. Material and Method

Subjects and tissue samples

To isolate ADSC, adult adipose tissues were obtained from five women who underwent gynecologic operation via laparotomy after receiving informed consent. To minimize individual variability, paired adipose tissues from visceral and subcutaneous fat were obtained from the same subjects. Subjects were 48 ± 2.4 yr of age (means ± SE; range 35 to 68 yr). The subject's body mass index (BMI) was 25 ± 0.7 kg/m^2 (means ± SE; range 21 to 30 kg/m^2). Tissue samples were collected for study as follows: In the operation room, adipose tissues retrieved from visceral and subcutaneous depots during surgery were immediately transferred into PBS containing FBS to isolate ADSC for further analysis.

Isolation and culture of adipose-derived stromal cells

Adipose tissue was washed in PBS to remove contaminating blood cells. Adipose tissue was cut and then

connective tissue and blood vessel was removed. The specimen was minced, and digested with collagenase type IA (Sigma, St. Louis, MO, USA) for 1 hour at 37 ° C. The pellet was collected by centrifugation at 1500rpm for 4 minutes and then treated with RBC lysis buffer (Lonza, Barsel, Switzerland) for 5 minutes at room temperature to remove blood cells. After centrifugation, the pellet was filtered through 40 μm mesh filter. The filtrate was centrifuged, and MACS® cell separation (Miltenyi Biotec, Bergisch Gladbach, Germany) was performed with CD31, CD45, FCR antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) to isolate negative cells for CD31 (endothelial cell marker) and CD45 (hematopoietic cell marker), and the obtained stromal vascular fraction (SVF) was plated onto 100-mm culture dish in complete culture medium [1:1 Dulbecco's modified Eagle's medium-Ham's F-12 (DMEM/F12) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% amphotericin-B]. The cells were cultured at 37 ° C in humidified atmosphere with 5% CO_2. After 3 days, nonadherent cells were removed. Confluent cells were trypsinized and expanded. Adipose-derived stromal cells (ADSC) were obtained by expansion of the adherent cells at

passage 2.

RNA preparation, microarray genechip processing and data analysis

Total RNA was extracted using the QIAGEN RNeasy Mini kit (Hilden, Germany), and amplified and labeled according to the Affymetrix (Santa Clara, CA) GeneChip Whole Transcript Sense Target Labeling protocol. The resultant labeled cDNA was hybridized to Affymetrix Human Gene 1.0 ST arrays. The scanned raw expression values were background-corrected, normalized, and summarized using robust multiarray averaging (Irizarry et al., 2003). The data were processed based on median polish normalization method using the GeneSpring GX 11.0 (Agilent technologies, CA). This normalization method aims to make the distribution of intensities for each array in a set of array the same. The normalization, and log transformed intensity values were then analyzed using GeneSpring GX 11.0. Fold change filters included the requirement that the genes be present in at least 210% for up/down-regulated genes. Principal component analysis (PCA) was performed by computing and then plotting the ten principal components. It can

be visualized these clusters in 3-dimensional space and got an impression on cluster size, integrity, and distribution.

Quantitative real-time PCR

ADSC was transferred into RNAlater (Ambion, Austin, TX) and stored at -75°C until assay and total mRNA of the ADSC was extracted using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). The cDNA was synthesized from total RNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The expression levels of specific genes in ADSCs were quantified by real-time PCR using iQ SYBR GREEN Supermix (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was run for 35 cycles at 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Data on melting curve were collected to check the PCR specificity. An endogeneous reference gene (GAPDH) was included for standardization and the mRNA level of each gene was normalized to GAPDH mRNA.

3. Result

Differential gene expressions in V-ADSCs and S-ADSCs

To identify the genes that are differentially expressed in subcutaneous ADSCs (S-ADSCs) and visceral ADSCs (V-ADSCs), the DNA micro-array of ADSCs at passage 2 from five subjects was analyzed. To evaluate the visualization of the relationships between the groups, the principal components of 10 samples were analyzed. PCA displayed the relationships between the S-ADSCs and V-ADSCs from five individuals. The S-ADSCs and V-ADSCs were generally similar, but the V-ADSCs and S-ADSCs from individuals 1 and 5 were separated (Figure 1). Of the 20,313 genes tested, the expression levels of 810 genes in the two groups statistically differed ($P < 0.05$). A total of 428 genes were upregulated in the V-ADSCs, and 382 genes in the S-ADSCs. The differentially expressed gene signature was enriched for the genes in the Gene Ontology biological processes of the metabolic process, lipid metabolism, development, angiogenesis, and ion transportation in the V-ADSCs, whereas the genes

involved in the transcription, development, and cellular metabolic processes were highly expressed in the S-ADSCs (Figure 2). The gene ontologies with significant differences ($p < 0.00001$) were selected (Table 2). The V-ADSCs showed the cholesterol biosynthetic process, negative regulation of the MAP kinase activity that regulates the differentiation of ADSCs, and negative regulation of cell proliferation. The signaling pathways are also shown in Table 3. The genes implicated in cholesterol biosynthesis (DHCR24, MVD, SQLE, SC4MOL, NSDHL, HMGCS1, ACAT2, and PDSS1) and triacylglyceride metabolism (ABHD5, MGLL, ALDH2, and LPPR4) were upregulated in the V-ADSCs. Although the genes in the S-ADSCs expressed related levels of sphingolipid metabolism, metabolism of glycerophospholilids and ether lipids, and metabolism of triacylglycerols, the differences in the expressions were low.

Among the genes related to cholesterol metabolism, ACAT2 encodes acyl-CoA : cholesterol acyltransferase, which is an enzyme that catalyzes cholesterol ester synthesis from cholesterol and long-chain fatty acyl-CoA (Rudel et al., 2005). HMGCS1, a gene code for HMG-CoA synthase-1, which converts acetyl-CoA and acetoacetyl-CoA to HMG-CoA, is a

precursor of cholesterol biosynthesis. The enzyme mevalonate pyrophosphate decarboxylase (MVD) catalyzes the conversion of mevalonate pyrophosphate into isopentenyl pyrophosphate in one of the early steps of cholesterol biosynthesis. Squalene epoxidase (SQLE) catalyzes the first oxygenation step in sterol biosynthesis and is thought to strongly influence the flux in this pathway. DHCR24 (24-dehydrocholesterol reductase) is required for the conversion of lanosterol to cholesterol. NSDHL (steroid dehydrogenase) and SC4MOL (sterol-C4-mehtyl oxidase) are involved in the sequential removal of two C-4 groups in post-squalene cholesterol biosynthesis.

As for the genes related to fatty acid metabolism, ABHD5 (abhydrolase domain-containing gene) activates adipose triglyceride lipase, which hydrolyzes triglyceride to yield diacylglyceride and fatty acids (Zimmermann et al., 2004). MGLL encodes a serine hydrolase of the AB hydrolase superfamily that catalyzes the conversion of monoacylglycerides to free fatty acids and glycerol.

Difference in the expressions of genes related to energy metabolism in the S-ADSCs and V-ADSCs

Of the 20,313 genes that were tested, 810 genes in the two groups showed statistically different expression levels ($P < 0.05$ and fold change ≥ 1.2). Ten genes were highly expressed between the V-ADSCs and S-ADSCs at $\geq$ a fourfold change with $P < 0.05$. Of these genes, three were associated with fat metabolism (TCF21, TM4SF1, and MMP3). TCF21 (transcription factor 21) and TM4SF1 (transmembrane 4 L six family member 1) were expressed more highly in the V-ADSCs than in the S-ADSCs, whereas MMP3 (matrix metallopeptidase 3) was overexpressed in the S-ADSCs (Table 4).

Confirmation of the different gene expressions via quantitative PCR

To confirm the micro-array analysis, real-time PCR was performed. Ten genes (TNC, MMP3, HAPLN1, MME, EYA, TCF21, TM4SF1, SLC14A1, PARM1, and PTN) that showed significant change were selected. Consistent with the micro-array data, TNC, MMP3, HAPLN1 and MME were significantly more highly expressed in the S-ADSCs than in the V-ADSCs.

Conversely, EYA, TCF21, TM4SF1, SLC14A1, PARM1, and PTN were more highly expressed in the V-ADSCs than in the S-ADSCs (*P < 0.05) (Figure 3).

Comparison of the genes in the S-ADSCs and the V-ADSCs from each patient

Donors 1 and 4 were normal and donors 2, 3, and 5 were obese. Therefore, the DEG was evaluated based on the patient profiles. A heat map represented the different expression patterns of the patient in the ADSCs that were isolated from the same location (Figure 4). Tables 5 and 6 show the differentially expressed genes. The gene lists of the S-ADSCs and V-ADSCs differed across the patients.

Table 1. Clinical features of study subjects. Subcutaneous and visceral adipose tissues were collected from 5 females.

	Age (yr)	Weight (kg)	BMI (kg/m^2)	Waist (mm)	Hip (mm)	W/H ratio	Diagnosis	Complication
Donor 1	45	54.9	21.2	720	900	0.8	Normal	Myoma uteri, Thyroid cancer
Donor 2	68	63.5	26.6	930	950	0.98	Central obese	Carcinosarcoma
Donor 3	47	62.2	23.5	922	1000	0.92	Central obese	Ovarian cancer
Donor 4	35	54.1	22.2	746	900	0.83	Normal	Cervical cancer
Donor 5	46	85.3	30.0	869	1063	0.82	Obese	Ovarian endometriosis
Mean±SE	48.2±5.4	64±5.7	24.8±1.6	837.4±44.1	962.6±31.2	0.87±0.03		

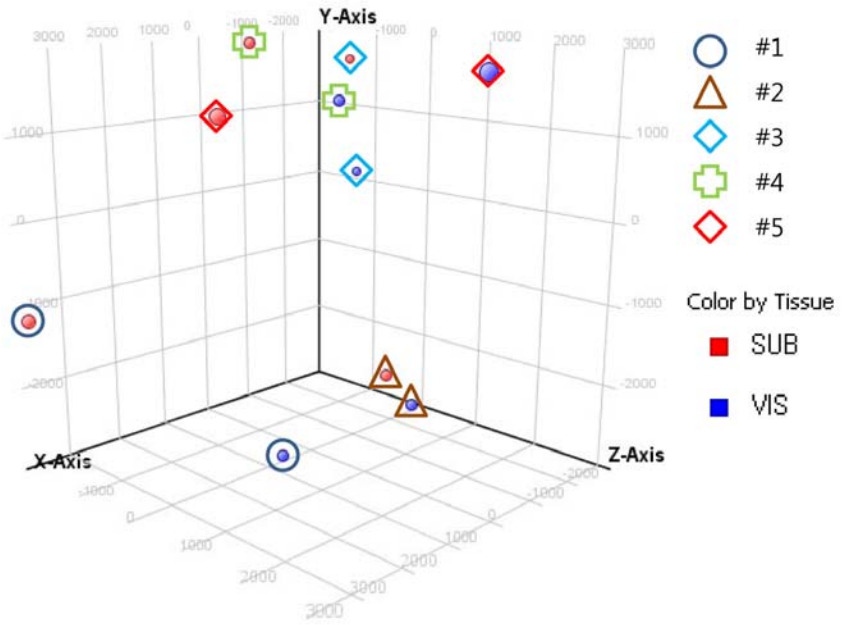
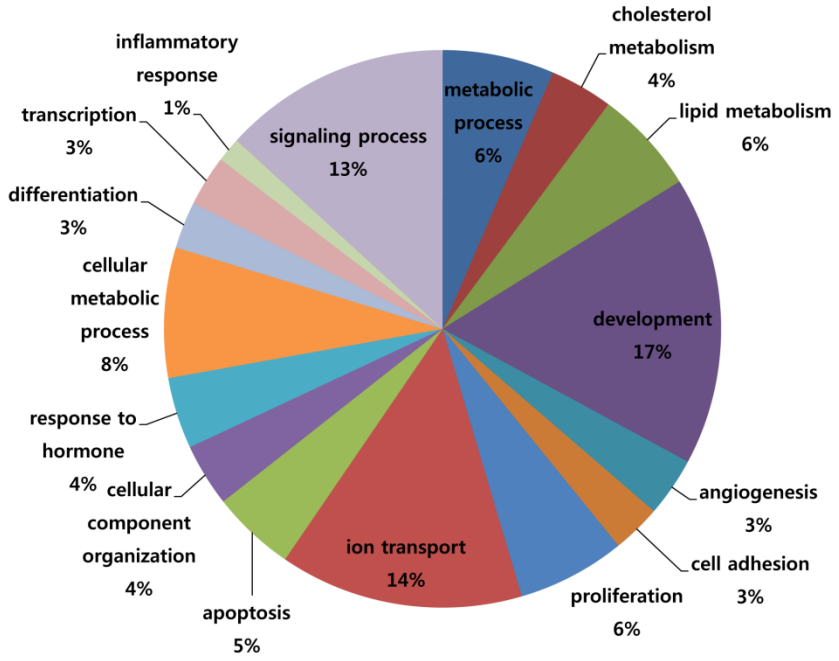


Figure 1. Principal component analysis (PCA) for each cell of origin using a data set. Principal component analysis identified variability of the gene expression profiles to be assessed by their proximity in three-dimensional space. PCA of the genes in stem cells from 2 individuals (#1 and #5) indicated that expression profiles of subcutaneous adipose-derived stromal cells differ substantially from those of visceral adipose-derived stem cells (X-Axis, first principal component: Y-Axis, second principal component: Z-Axis, third principal component).

A. Biological processes of up-regulated genes in V-ADSC



B. Biological processes of up-regulated genes in S-ADSC

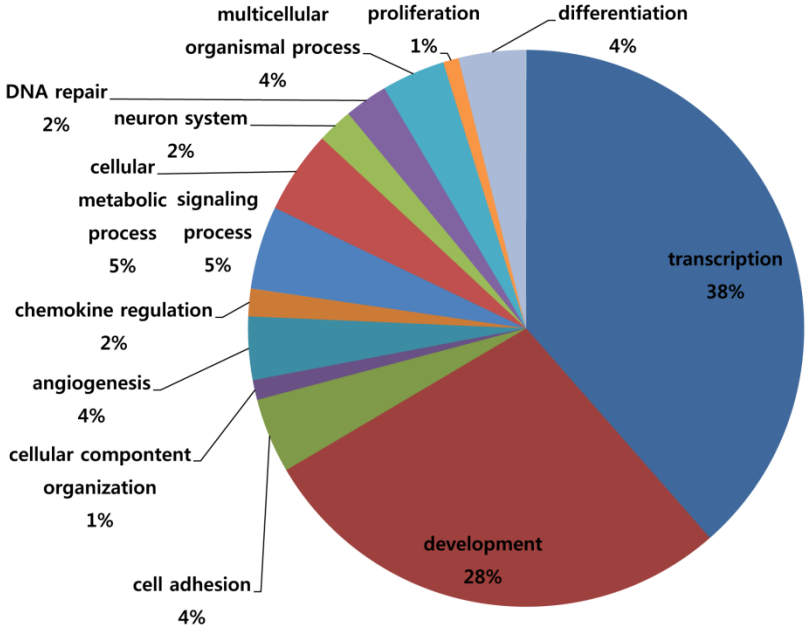


Figure 2. Pie diagram of biological processes of gene ontology that were differentially expressed genes between visceral adipose-derived stromal cell (V-ADSC) (A) and subcutaneous adipose-derived stromal cell (S-ADSC) (B). The gene signature was enriched for genes in gene ontology biological processes of cholesterol, lipid metabolism, development, signaling process and ion transport in V-ADSC. S-ADSC represented genes involved in transcription and development.

Table 2. Gene ontology (GO) of differentially expressed genes in V-ASC and S-ASC ($p<0.0001$).

category	Total gene	Over lap	p-value
Up-regulated in V-ASC			
metabolic process	2421	55	$1.16X10^{-11}$
cholesterol biosynthetic process	42	8	$2.15\ X10^{-9}$
sterol biosynthetic process	30	7	$4.89X10^{-9}$
lipid metabolic process	372	17	$2.27X10^{-8}$
lipid biosynthetic process	128	10	$1.48X10^{-7}$
multicellular organismal development	1146	28	$5.47X10^{-7}$
blood coagulation	477	17	$7.70X10^{-7}$
response to glucocorticoid stimulus	95	4	$4.05\ X10^{-6}$
negative regulation of MAP kinase activity	30	5	$4.97X10^{-6}$
cell adhesion	686	19	$6.99\ X10^{-6}$
steroid biosynthetic process	85	7	$7.89X10^{-6}$
negative regulation of cell proliferation	399	14	$8.79\ X10^{-6}$
oxidation-reduction process	840	21	$1.09\ X10^{-5}$
BMP signaling pathway	60	6	$1.17\ X10^{-5}$
apoptosis	778	19	$3.94\ X10^{-5}$
Ureteric bud development	46	5	$4.28X10^{-5}$
ion transmembrane transport	540	15	$6.34\ X10^{-5}$
transmembrane transport	831	19	$9.40\ X10^{-5}$
Up regulated in S-ASC			
transcription, DNA-dependent	2265	58	$6.69X10^{-18}$
regulation of transcription, DNA-dependent	2872	61	$4.84X10^{-15}$
multicellular organismal development	1146	37	$1.74X10^{-14}$
embryonic limb morphogenesis	69	7	$6.63X10^{-7}$
cell adhesion	686	18	$2.69X10^{-6}$
embryonic skeletal system morphogenesis	60	6	$4.64X10^{-6}$
chemorepulsion involved in postnatal olfactory bulb interneuron migration	2	2	$5.13X10^{-5}$
negative regulation of transcription from RNA polymerase II promoter	506	13	$8.35X10^{-5}$

Total gene: number of all genes associated to the gene ontology term; overlap: number of differentially expressed genes associated to the gene ontology term

Table 3. Pathway analysis of up-regulated genes in visceral adipose-derived stromal cells. The metabolic pathway annotating categories showed significant enrichment in genes expressed in V-ADSC.

category	Gene	p-value
Biosynthesis of cholesterol	DHCR24, MVD, SQLE, SC4MOL, NSDHL, HMGCS1	$2X10^{-3}$
Mevalonate pathway	ACAT2, MVD, PDSS1, HMGCS1	$2.3X10^{-3}$
Metabolism of triacylglycerols	ABHD5, MGLL, ALDH2, LPPR4	$3.9X10^{-2}$

Table 4. Gene list of the highest fold changes in visceral adipose-derived stromal cell (V-ADSC) and subcutaneous adipose-derived stromal cell (S-ADSC) ($p<0.05$, $FC>4$).

Gene bank Assession NO.	Symbol	Gene	Fold change	p-value
		Up in S-ADSC		
NM_002160	TNC	tenascin C	8.00	$4.98X10^{-5}$
NM_002422	MMP3	matrix metallopeptidase 3	7.24	0.01
NM_001884	HAPLN1	hyaluronan and proteoglycan link protein 1	5.78	0.004
NM_007288	MME	membrane metallo-endopeptidase	4.80	0.004
		Up in V-ADSC		
NM_004100	EYA4	eyes absent homolog 4 (Drosophila)	5.98	$1.86X10^{-4}$
NM_003206	TCF21	transcription factor 21	5.44	0.001
NM_014220	TM4SF1	transmembrane 4 L six family member 1	5.43	0.03
NM_001128588	SLC14A1	solute carrier family 14 (urea transporter), member 1 (Kidd blood group)	4.60	0.01
NM_015393	PARM1	Prostatic androgen repressed message 1	4.58	0.003
NM_002825	PTN	pleiotrophin	4.00	0.009

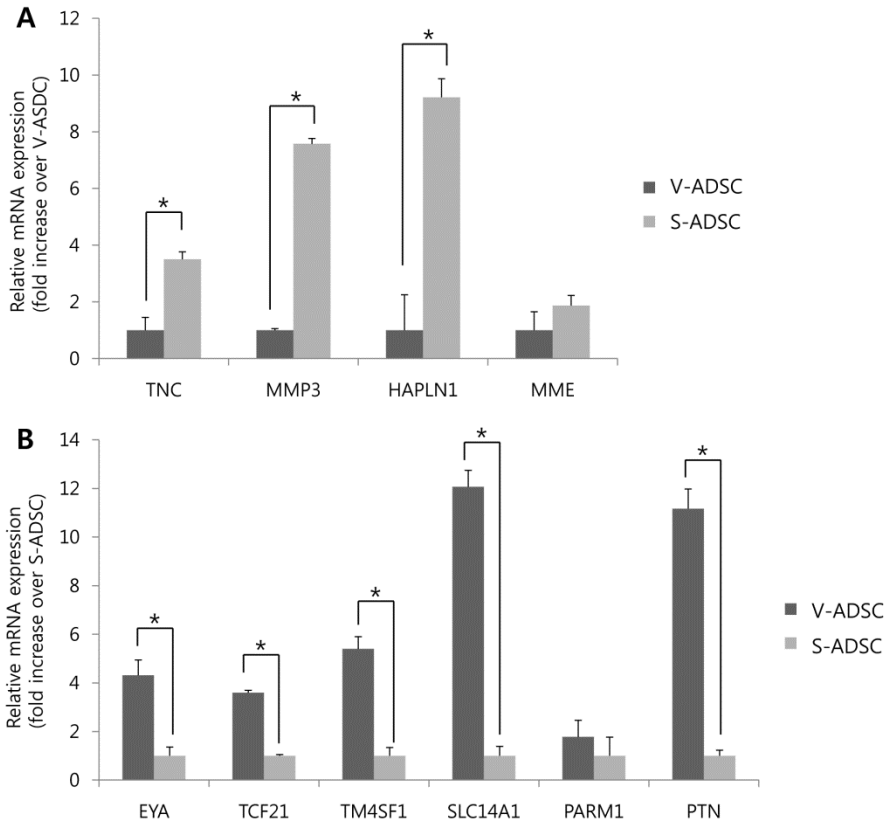


Figure 3. Differentially expressed mRNA levels of TNC, MMP3, HAPLN1, MME (A), EYA, TCF21, TM4SF1, SLC14A1, PARM1 and PTN (B) between V-ADSC and S-ADSC which were evaluated by quantitative real-time PCR. Transcriptional levels of these genes were different between S-ADSC and V-ADSC with statistical significance (*$p<0.05$)

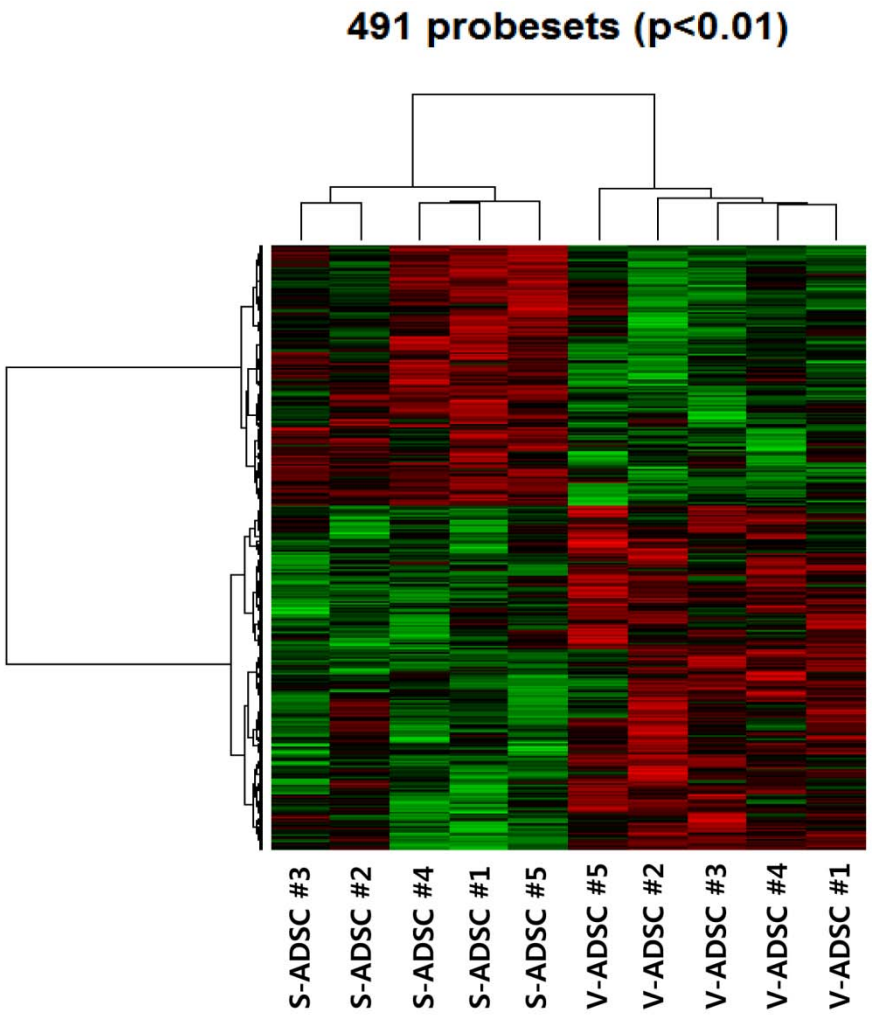


Figure 4. Differentially expressed genes identified between adipose derived stromal cells from visceral fat depot (V-ADSCs) and those from subcutaneous fat depot (S-ADSCs) (Fold change $\geq$ 2, $P < 0.01$). Red and green colors indicate high and low expression, respectively.

Table 5. Differentially expressed genes between S-ADSC and V-ADSC from each patient.

Sets of comparison	Up-regulated	Down-regulated	total
#1 V- vs. S-ADSC	290	161	451
#4 V- vs. S-ADSC	73	55	128
#2 V- vs. S-ADSC	112	150	262
#3 V- vs. S-ADSC	94	64	158
#5 V- vs. S-ADSC	182	105	287

Table 6. List of genes that have the highest difference of fold change in each patient.

#1				#4			
V-ADSC	FC	S-ADSC	FC	V-ADSC	FC	S-ADSC	FC
MALL	4.3	STMN2	4.9	TCF21	3.4	HAPLN2	3.4
SHCBP1	4.3	MME	3.3	EYA4	3.3	TNC	3.3
DUX4	4.3	ADH1B	3.1	CNTN4	3.1	C5orf31	3.0
TM4SF1	4.2	FIBIN	3.1	SLC22A3	3.0	LOC100129901	3.0
HHIP	4.1	DRD5	3.0	ANKRD1	2.8	MME	2.7

#2				#3				#5			
V-ADSC	FC	S-ADSC	FC	V-ADSC	FC	S-ADSC	FC	V-ADSC	FC	S-ADSC	FC
BCHE	3.4	TNC	3.3	TM4SF1	2.9	MMP3	4.2	TSPAN8	4.6	NRN1	5.3
LINGO2	3.4	MMP3	3.2	LPPR4	2.9	HAPLN1	3.3	VCAM1	4.2	MMP3	4.2
RGS7BP	3.2	ENPP1	2.7	PTN	2.7	TNC	3.0	HGF	4.1	ENPP2	4.1
DKFZP	3.1	HHIP	2.6	EYA4	2.6	RNU1A	2.6	ANXA10	3.9	SFRP2	3.6
SFRP2	2.9	MOXD1	2.5	CFB	2.6	STMN2	2.4	HSD3B1	3.8	DPT	3.3

4. Discussion

Since adipose tissues from different regions have been studied as to their responsibility for metabolic disease, few reports have introduced stromal cells from subcutaneous and visceral adipose tissues that contribute to the production of mature adipocytes. To the authors' knowledge, this is the first study that profiled gene expression in human S-ADSCs and V-ADSCs from a single individual, using a large-scale micro-array assay.

Different gene expression profiles were found between the S-ADSCs and the V-ADSCs. As a result, more than 800 genes (P value < 0.05) were identified as differentially expressed between the S-ADSCs and the V-ADSCs. In addition, the principal component analysis revealed the separation of the S-ADSCs and the V-ADSCs, but not in all the individuals who had subcutaneous fat that basically differed from visceral fat. Gene clustering was presented by biological function in the Gene Ontology, which provides bio-informatics tools for micro-array data analysis. The Gene Ontology analysis of the array results revealed that the overexpressed genes in the V-ADSCs were associated with biological

processes that included cholesterol biosynthesis, negative regulation of the MAPK activity that regulates differentiation into adipocytes, and negative regulation of cell proliferation, unlike those in the S-ADSCs. This observation is in accordance with the finding that the V-ADSCs poorly proliferated and differentiated *in vitro*, unlike the S-ADSCs. Also numerous studies have highlighted the strong correlation of hypertrophied adipocytes with adipocyte dysfunction in obesity and cholesterol imbalance (Krause and Hartman, 1984). These characteristics of visceral adipose tissue may explain why genes involved in cholesterol and lipid metabolism were upregulated in the V-ADSCs. Moreover, the cholesterol content of lipid droplets has been known to be closely related to the droplets' triglyceride content (Dugail et al., 2003). In line with this fact, the genes involved in triacylglyceride metabolism were expressed more in the V-ADSCs.

Among the highly expressed genes, the genes involved in energy metabolism were focused on in this study. Five genes were identified as differentially expressed in the V-ADSCs and S-ADSCs. TCF21 and TM4SF1 were highly expressed in the V-ADSCs, whereas MMP3 was overexpressed in the S-ADSCs.

TCF21 (transcription factor 21) is a basic-helix-loop-helix transcription factor that is highly expressed in the mesenchyme of multiple organs (Cui et al., 2003). Its mRNA levels in diabetic db/db mice were significantly upregulated (Makino et al., 2006), which suggests that it could participate in adipose tissue differentiation *in vivo*.

Another upregulated gene in the V-ADSCs corresponded to transmembrane 4 L six family member 1 (TM4SF1). TM4SF1 is a member of the transmembrane 4 superfamily, also known as the tetraspanin family. It has been reported to activate cell signaling pathways. The precise biological properties of this superfamily largely remain unknown, however. Recent studies have suggested that TM4SF1 may be involved in the development of obesity and insulin resistance. It was overexpressed in large adipocytes that resist insulin, unlike in small adipocytes (Jernas et al., 2006). In addition, it showed a higher expression level in visceral adipose tissue from obese men than in subcutaneous fat from the same individuals (Gabrielsson et al., 2003).

In contrast, the S-ADSCs showed a different pattern of gene expression, which included overexpression of matrix metallopeptidase 3 (MMP3). MMP3 is involved in extracellular

matrix degradation in normal physiological processes such as embryonic development and tissue remodeling, as well as in disease processes. In addition to MMP3' s tissue remodeling role, it is considered to negatively control obesity. MMP3 inhibition resulted in extensive fat accumulation and more hypertrophic cells in mice (Maquoi et al., 2003). In humans, MMP3 was found to have been downregulated in pre-adipocytes and stromal vascular cells from obese subjects, unlike those from non-obese subjects (Traurig et al., 2006).

Also, there were significant differences across the patients, which are important to study to come up with individual pharmaceutical treatment regimens. They will thus be studied further to obtain more specific gene profiles.

These results indicate that the V-ADSCs and S-ADSCs are biologically distinct. Consistent with the findings from previous studies that visceral fat is closely associated with metabolic disorder, the findings from this study showed that visceral and subcutaneous adipose tissues have distinct properties and origins.

CHAPTER 3.

CHARACTERIZATION OF ADIPOSE-DERIVED STROMAL CELLS

1. Introduction

Adipose tissue is an attractive reservoir of progenitor cells. It contains the stromal vascular fraction (SVF), which can be readily isolated via enzymatic dissociation (Zuk et al., 2002). This SVF from adipose tissue consists of a heterogeneous mixture of cells that include endothelial cells, smooth muscle cells, pericytes, leukocytes, and pre-adipocytes. In addition to these cells, the SVF contains an abundant population of multipotent adipose-tissue-derived stromal cells (ADSCs) (da Silva Meirelles et al., 2008; Bailey et al., 2010).

In 1964, Martin Rodbell was the first to report a method of in vitro isolation of mature adipocytes and adipogenic progenitors from rat fat (Rodbell, 1964). Katz, Zuk, et al. were the first to show an SVF population isolated from human lipoaspirates that contained cells with multilineage potential (Katz et al., 1999; Zuk et al., 2001). They isolated the adipose stromal cells from the liposuction aspirates using the collagenase digestion method, and subsequently allowed the ADSCs to adhere to the plastic surface of culture flasks. This method is still basically used.

With respect to the cell source for regenerative medicine, the characterization and comparison of ADSCs from different depots have been reported (Fraser et al., 2007; Jurgens et al., 2008; Schipper et al., 2008). ADSCs from subcutaneous and visceral depots have different functional properties and charicteristics. The underlying mechanism of this difference is not yet clear, though.

In this study, ADSCs were established from subcutaneous and visceral depots. Because the SVF contains diverse cells, endothelial- and hematopoietic-origin cells (CD31 and CD45) were removed and more homogeneous cells were collected, which indicates that $CD31^-/CD45^-$. Then the immunotypes of the ADSCs and their differentiation potentials were identified.

2. Materials and Methods

Isolation and culture of adipose-derived stromal cells

Adipose tissue was washed in PBS to remove contaminating blood cells. Adipose tissue was cut and then connective tissue and blood vessel was removed. The specimen was minced, and digested with collagenase type IA (Sigma, St. Louis, MO, USA) for 1 hour at 37 ° C. The pellet was collected by centrifugation at 1500rpm for 4 minutes and then treated with RBC lysis buffer (Lonza, Barsel, Switzerland) for 5 minutes at room temperature to remove blood cells. After centrifugation, the pellet was filtered through 40 μm mesh filter. The filtrate was centrifuged, and MACS® cell separation (Miltenyi Biotec, Bergisch Gladbach, Germany) was performed with CD31, CD45, FCR antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) to isolate negative cells for CD31 (endothelial cell marker) and CD45 (hematopoietic cell marker), and the obtained stromal vascular fraction (SVF) was plated onto 100-mm culture dish in complete culture medium [1:1 Dulbecco's modified Eagle's medium-Ham's F-12

(DMEM/F12) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% amphotericin-B]. The cells were cultured at 37 °C in humidified atmosphere with 5% CO_2. After 3 days, nonadherent cells were removed. Confluent cells were trypsinized and expanded. Adipose-derived stromal cells (ADSC) were obtained by expansion of the adherent cells at passage 3.

Flow cytometry analysis

Immunophenotypical analysis of S-ADSC as well as V-ADSC was performed by using FITC- and PE-conjugated antibodies: CD31, CD34, CD45, CD73, CD90, CD105, and their respective isotype control mAbs (BD PhaMingen, San Diego, CA). CD31 is an endothelial marker, and CD34 and CD45 are hematopoietic markers. CD73, CD90 and CD105 were used as stromal markers.

For flow cytometry, ADSC were detached using 0.05% trypsin-EDTA, washed and resuspended at a concentration of 10^5 cells/ml. Cells (10^5) were incubated with the 2 μl antibodies in 100 μl PBS containing 2% FBS at 4 °C for 20 min, washed with 1ml of PBS containing 2% FBS, centrifuged 5000rpm at

4 °C for 2 min, and resupended with 1 μg/ml 7-AAD for 10min. Ten thousand events were acquired for FACS-caliber flow cytometry using CELLquest acquisition software.

In vitro cell differentiation

ADSC were induced to differentiate into three different types of cells, including adipocytes, osteocytes and chondrocytes, to evaluate the differentiating potentials of ADSCs. Cells were plated at a density of approximately 1.5 X 10^4 cells/cm^2. Cells were cultured in specific media for differentiation for 3 weeks, and the media were changed every 3 days.

For adipogenic differentiation, cells were incubated in adipogenic media (DMEM/F12 containing 10% FBS, 0.5mM isobutylmethylxanthine, 5nM indomethacin and 500nM dexamethasone). After 3 weeks, differentiated cells cultured in adipogenic media were fixed for 1 hour with 4% paraformaldehyde, washed with 60% isopropanol, and stained with Oil Red O for 10 min to detect intracellular lipid accumulation which suggests adipocyte differentiation. For destaining, 100% isopropanol was treated for 15min, and then

the optical density (OD) of the destained solution was measured at 450 nm with an ELISA.

For osteogenic differentiation, cells were incubated for 3 weeks in osteogenic media (DMEM/F12 containing 10% FBS, 1nM dexamethasone, 10mM β-glycerolphosphate and 50μM ascorbate-2-phosphate). At the indicated time point, cells cultured in osteogenic media were washed with PBS, fixed 4% paraformaldehyde for 10 min and rinsed with PBS. For detection of bone formation, cells were incubated in alizarin red S (Sigma-Aldrich) for 10 min.

For chondrogenic differentiation, ADSC were seeded at a density of 2 X 10^4 cells per pellet in 15-ml conical tubes with chondrogenic media (high glucose DMEM containing 10% FBS, 500ng/ml BMP-6, 10ng/ml TGF-β3, 100nM dexamethasone, 50μg/ml ascorbate-2-phosphate, 40μg/ml proline, 100μg/ml pyruvate and 1X ITS liquid media). Cells were centrifuged at 1500rpm for 4 min to the bottom of the tubes and allowed to form compact cell pellets at 37°C under 5% CO_2. Cells were maintained as a small pellet in the chondrogenic differentiation medium for 3 weeks. At the indicated time points, differentiated cells were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections of the pellet were cut and

stained with Toluidine Blue to detect sulfated glycosaminoglycans.

Adipogenic differentiation potential

Differentiated cells cultured in adipogenic media were fixed for 1 hour with 4% paraformaldehyde, washed with 60% isopropanol, and stained with Oil Red O for 10 min to detect intracellular lipid accumulation which suggests adipocyte differentiation. For destaining, 100% isopropanol was treated for 15min, and then the optical density (OD) of the destained solution was measured at 450 nm with an ELISA.

Proliferation assay

ADSC were seeded at $5X10^{3}$ cells cm^{-2} in a 12-well plate and incubated in basal medium. The cells were trypsinized at days 2, 4, and 7 and cell number was counted by hemocytometer. Average number of cells per well was calculated and a growth curve was generated.

3. Result

Isolation of ADSCs from adipose tissue

Human subcutaneous and visceral adipose tissues were obtained from a total of five donors [five females: age, 48±2.4 years (mean±SE) and range, 35–68 years] who had undergone surgery (Table 1). The adipose tissue samples were digested with collagenase, and then filtered and centrifuged. The bottom pellet that contained the SVF was collected. The SVF was negative for CD31 and CD45.

Immediately after the separation, the $CD31^-/CD45^-$ and $CD31^+/CD45^+$ cells were resuspended and cultured under standard ADSC culture conditions. The freshly isolated $CD31^-/CD45^-$ cells attached themselves to the plastic surface and showed a fibroblastic–like morphology. The $CD31^+/CD45^+$ cells from the subcutaneous and visceral adipose tissues proliferated poorly (data not shown). The ADSCs were cultured until passage 3 to characterize them.

Expression of cell-surface antigens

The cell surface antigen profiles of the S-ADSCs and the V-ADSCs were observed and isolated from the adipose tissues of the subcutaneous and visceral compartments. Although the donors hardly differed, their ADSCs showed similar immunophenotypical profiles. The isolated V-ADSCs and S-ADSCs were both positive for stromal cell markers that included CD73, CD90, and CD105, and negative for the endothelial cell marker CD31 and for hematopoietic cell markers that included CD34 and CD45 (Figure 5).

Multilineage differentiation potential

The differentiation potential of the ADSCs was analyzed *in vitro* under proper conditions for the adipogenic, osteogenic, and chondrogenic lineages. After three weeks of adipogenic differentiation, the S- ADSCs (Figure 6A) and the V-ADSCs (Figure 6D) showed accumulation of lipid-rich vacuoles, as detected via Oil Red O staining. When osteogenic differentiation was induced in the S-ADSCs (Figure 6B) and the V-ADSCs (Figure 6E), their populations formed a mineralized matrix that

was detected via Alizarin Red S staining. The S-ADSCs (Figure 6C) and the V-ADSCs (Figure 6F) were also capable of chondrogenic differentiation, and accumulated a sulfated proteoglycan-rich matrix that was visualized via Toluidine Blue staining.

Adipogenic differentiation potential

The adipogenic differentiation ratios of the S-ADSCs and the V-ADSCs were examined. After three weeks of induction, the dye was extracted and its optical density was determined. Although a slight difference was found between the individuals, the S-ADSCs showed more significant staining than the V-ADSCs (Figure 7).

Proliferation assay

Proliferation was expressed as the number of cells counted in Days 2, 4, 6, and 8. Figure 8 compares the proliferation rates of S-ADSCs and the V-ADSCs from the same subject. The S-ADSCs proliferated at a higher rate than the V-ADSCs.

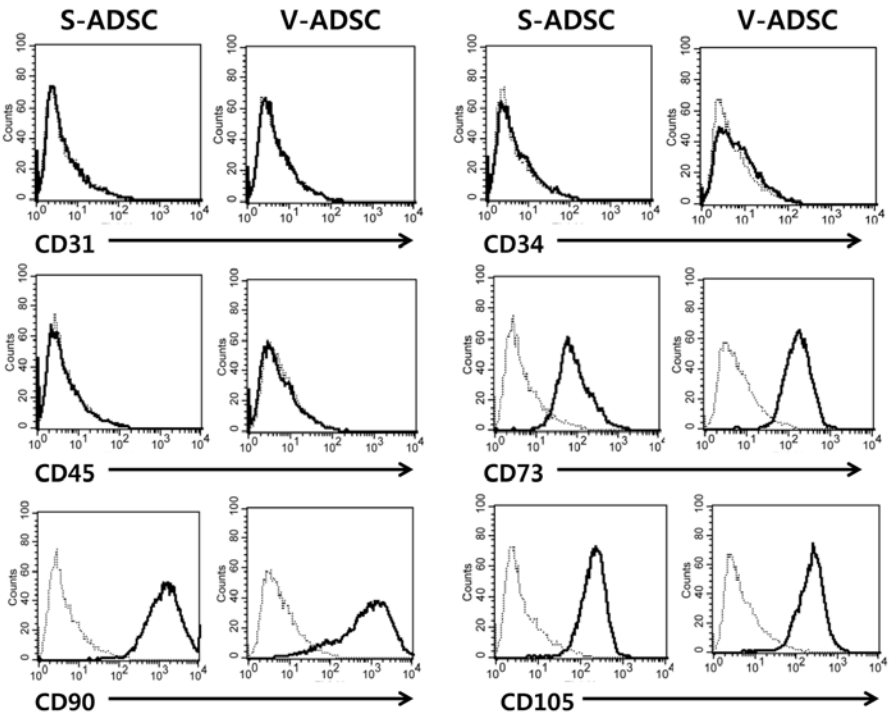


Figure 5. Immunophenotypical characterization of adipose-derived stromal cells (ADSC). FACS analysis of S-ADSC and V-ADSC revealed a similar positivity for the indicated surface markers in both populations. Black and dotted line represents specific and control antibody staining, respectively. Cell counts and fluorescence intensity are indicated in y-axis and x-axis.

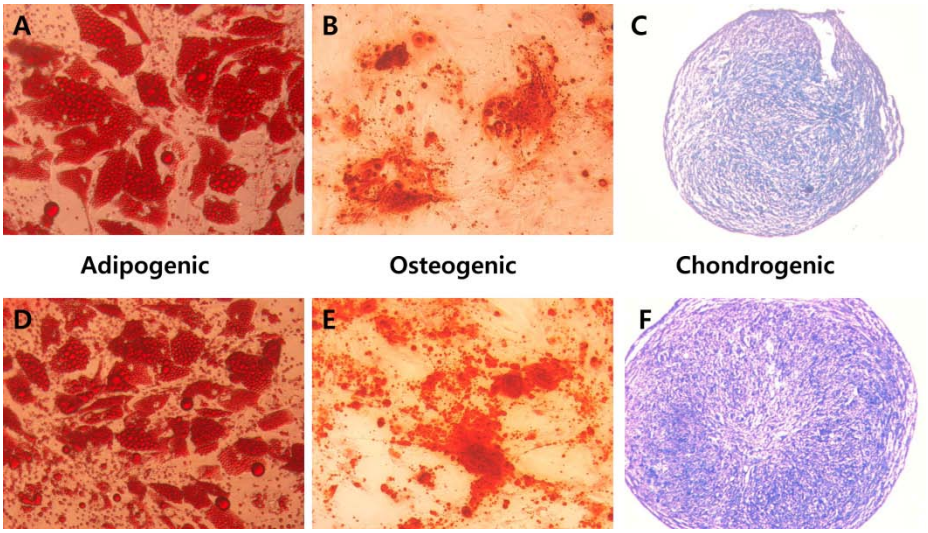


Figure 6. Differentiation of adipose-derived stromal cells from subcutaneous depots (S-ADSC) and those from visceral depots (V-ADSC) into adipogenic, osteogenic and chondrogenic lineages. After 3 weeks of culture in induction media, differentiated cells were stained with Oil Red O, Alizarine Red S and Toluidine Blue to detect lipid accumulation, bone formation and sulfated glycosaminoglycans, respectively. Both S-ADSC and V-ADSC showed adipogenic (A, D), osteogenic (B, E) and chondrogenic (C, F) differentiations. Magnification: adipogenic lineage, X400; chondrogenic and osteogenic lineages, X200.

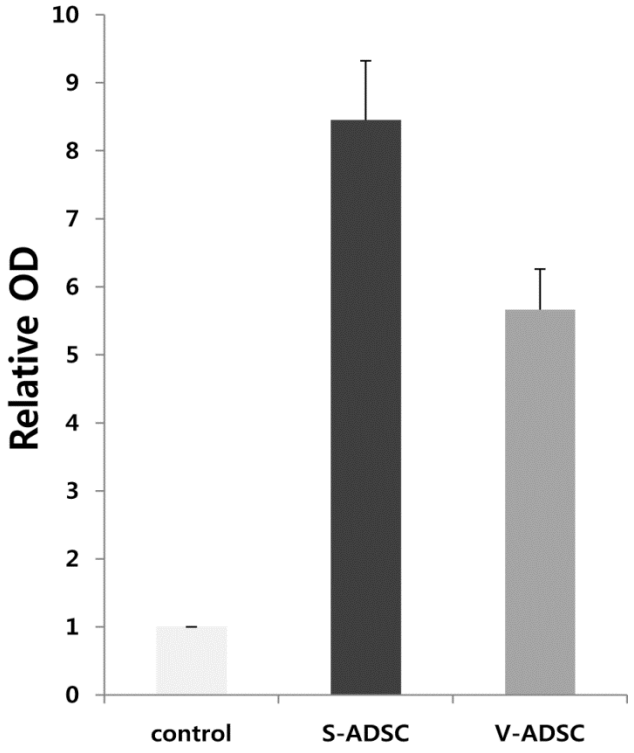


Figure 7. Assessment of adipose differentiation by staining depends on the rate of lipid accumulation. ADSC was cultured in adipogenic differentiation conditions. Three weeks after induction, lipid accumulation was determined by Oil red O staining. The dye retained by lipid vacuoles was measured by determination of the optical density at 450nm and is shown on the y-axis. The graph shows the representative results of five subjects, each performed triplicate, with S-ADSC and V-ADSC.

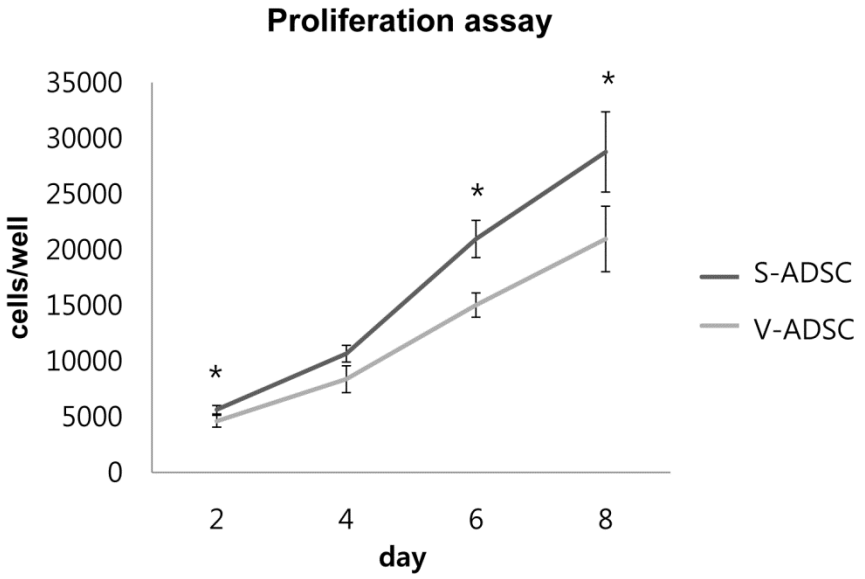


Figure 8. Regional variation in proliferation potential of subcutaneous (S-ADSC) and visceral adipose-derived stromal cells (V-ADSC). Both ASCs of 5 X 10^3 cells cm^{-2} were seeded in a 12-well plate and incubated. ADSC were detached at indicated day and cell numbers were counted by hemocytometer. S-ADSC had greater proliferation rate than V-ADSC. The graph shows the representative results of five subjects, each performed triplicate, with S-ADSC and V-ADSC (*$p<0.05$).

4. Discussion

This paper reports the isolation and characterization of *in vitro* primary ADSCs from visceral and subcutaneous adipose tissues.

Primary ADSCs contain various other cells, because conventional methods of isolation of ADSCs have relied on the adhesion of the cells to plastic. In an earlier study, when $CD45^+$ cells were removed from the SVF, the remaining cells were separated into two populations based on the expression of CD31, a marker of endothelial cells (Boquest et al., 2005). In this study, ADSCs were obtained by removing the $CD31^+/CD45^+$ cells to reduce the contamination of the endothelial and hematopoietic cells. This was manifested by the adhesion of the cells of the $CD31^-/CD45^-$ phenotype to plastic, the change in their morphology to a fibroblastoid morphology, and their extensive proliferation under standard ADSC culture conditions.

The S-ADSCs had a greater ability to proliferate than the V-ADSCs. Similarly, pre-adipocytes from subcutaneous adipose tissue have been reported to be more proliferative than those from visceral adipose tissue (Van Harmelen et al., 2004;

Joe et al., 2009). In addition, the comparison of the differentiation potentials showed that the S-ADSCs were more significantly differentiated into mature adipocytes, which is consistent with the results of other studies (Tchkonia et al., 2005; Toyoda et al., 2009). It was demonstrated that several stem cells were related to the increased adipogenic differentiation (Aguena et al., 2012). Therefore, it can be said that subcutaneous adipose tissue can expand the number of cells that can become adipocytes. This explains that new and smaller adipocytes have a high ability to store free fatty acids and triglycerides, which will prevent their deposition in non-adipose tissue (Per M&in and Lars Sj6strbm, 1992; Vikram, 2003). On the other hand, V-ADSCs had a low propensity to proliferate and differentiate. These properties might make visceral adipocytes hypertrophic and dysfunctional. This means that S-ADSCs, which extensively proliferate and differentiate, are appropriate sources of regenerative medicine.

The findings from this study contribute to the characterization of the ADSCs in adipose tissue. The establishment of well-characterized ADSCs will allow an *in vitro* study of the molecular mechanism, the alterations of which may affect the process of adipogenesis and may result in

metabolic disease. According to the proliferation and differentiation of S-ADSCs, they may be ideal sources of regenerative medicine.

CHAPTER 4.

GENERAL DISCUSSION

AND

CONCLUSION

There has been increased interest in adipose derived stromal cells for tissue engineering application. Adipose tissue has proven to serve as an abundant, accessible and rich source of cells with multipotent properties for regenerative medical application. However, ADSC from subcutaneous and visceral depots have functional difference and property.

In chapter 2, we isolated in vitro primary cultures of ADSC obtained from subcutaneous and visceral adipose tissue from the same subject. We processed DNA microarray analysis comparing S-ADSC and V-ADSC. We found that the gene expression profile was clearly different in S-ADSC and V-ADSC, showing 810 genes was significantly different ($p < 0.05$). These results indicate that they have different properties even in adipose derived stromal cells consisting of adipose tissue. Furthermore, we focused on energy metabolism related genes. In the expression of V-ADSC, two genes overexpressed involved in dysregulated lipid metabolism and metabolic disorder. However, S-ADSC was upregulated one genes that was related to protective role for obesity. Different molecular mechanism whose dysregulation may underlie different metabolic dysfunctions.

In chapter 3, we characterized in vitro primary ADSC

obtained from subcutaneous and visceral adipose tissue from the same subject. By use of different technique, we provide experimental results that they share similar morphology and immunophenotypical properties, as well as multilineage differentiation potential. However, S-ADSC had higher potential to proliferate and differentiate into adipocytes.

In conclusion, we demonstrated the differential expression profiles of genes between ADSC derived from different anatomic regions. Given that S-ADSC and V-ADSC represent different cell characteristics from biochemical perspectives, our studies provide additional evidence of significant differences in the expression patterns between S-ADSC and V-ADSC. Taken together, adipocytes in different depots could be fundamentally the different in their properties, and contribute variation in inherent properties. Our findings provide further insights into understanding fat accumulation and distribution and therapeutic targets against metabolic disorders, such as obesity and insulin resistance.

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SUMMARY IN KOREAN

지방조직은 내장지방과 피하지방, 2가지로 나뉜다. 이 두 지방조직은 서로 다른 특징과 질병 위험도를 가지고 있다. 내장지방 비만은 피하지방 비만보다 대사질환에 더 밀접한 관련이 있는 것으로 알려져 있다. 여러 연구는 피하지방과 내장지방으로부터 분리한 지방세포와 지방전구세포의 유전자 발현을 분석하였고 해부학적 위치에 따라 다른 유전자 발현을 보였다.

첫번째로, MACS를 이용하여 지방 유래 기질세포를 분리하였다. 유전자 발현을 분석하기 위해 $CD31^-/CD45^-$ 세포가 분리되었고 배양되었다. 피하지방과 내장지방으로부터 분리된 기질세포의 유전자 발현 분석은 20,313개의 유전자중의 810개의 유전자가 서로 다르게 발현된다는 것을 보여주었다 ($P < 0.01$). 유의적으로 발현되는 유전자들은 다양한 유전자 ontology에 관련되어있었다.

다르게 발현되는 유전자 가운데, 에너지 대사에 관련된 다섯 개의 유전자에 집중하였다. V-ADSC에서는 TCF21, TM4SF1 이 과발현 되어있었고, 반면에 MMP3는 S-ADSC에서 높게 발현되어있었다. V-ADSC에서 높게 발현된 TCF21, TM4SF1은 잘못 조절된 지방대사와 관련되어 있었지만 S-ADSC에서 발현된 MMP3는 비만을 방지하는 역할에 관련이 있었다.

그 다음으로, 분리된 세포, $CD31^-/CD45^-$ (ADSC), 의 characterization 이 수행되었다. CD31, CD45, CD34, CD73, CD90, CD105을 사용해 피하지방과 내장지방으로부터 분리된 ADSC의 특징을 조사하였고 지방세포, 조골세포, 연골세포로 분화하는 성질을 평가하였다. 면역표현형은 ADSC가 중간엽 줄기세포의 일반적인 마커들을 갖는 것을 보여주었다. S-ADSC와 V-ADSC 모두 줄기세포의 성질을 보여주는 지방, 조골, 연골세포로 분화되었다. 하지만 증식능과 지방세포로 분화되는 능력은 S-ADSC가 V-ADSC보다 더 뛰어났다.

이 결과는 S-ADSC와 V-ADSC에서의 다른 유전자 발현 패턴을 보이고 피하지방과 내장지방의 본질적인 차이를 설명할 수 있다는 것을 보여준다. 게다가, 에너지 대사에 관련된 유전자들을 고려해볼 때, 피하지방과 내장지방의 지방세포는 그들의 특성에서 근본적으로 차이가 있을 것이다. 지방유래 기질세포의 차이는 분자적 메커니즘을 이해하는데 도움이 될 것이며 더 나아가 대사질환의 치료적 표적을 제공할 수 있을 것이다.

감사의 글

새로운 꿈을 가지고 대학원에 입학한 것이 얼마 안 된 것 같은데 벌써 졸업이라니 한편으로는 뿌듯하고 한편으로는 아쉬움이 많이 남습니다.

먼저 부족한 저에게 아낌없는 충고와 가르침을 주신 임정묵 교수님께 진심으로 감사 드리며 학위기간 중 제가 선택한 공부에 흥미를 느끼도록 해 주신 한재용 교수님, 송용상 교수님께도 감사의 말씀을 전합니다.

학문적인 내용을 넘어서 행복하고 즐거운 대학원 생활을 만들어주신 실험실 가족들, 안지연, 공승표 교수님, 장미, 김길아, 이재희, 최정규 박사님, 오서진 언니, 최준희 오빠, 이채현 언니, 염경은 언니, 김보연 언니, 안종일 오빠, 김화영 언니, 이아란 언니, 윤정기 오빠, 조윤미 언니, 이명욱 오빠, 박영현 오빠, 권효숙 언니, 고동우, 차가영 후배님 모든 분들께 감사 드립니다. 의대 실험실 식구들인 곽혜란 언니, 김영민, 이현주, 서정아, 문희선, 송지영, 홍윤희, 특히 저에게 도움 많이 주신 김미경 선생님 정말 감사 드립니다.

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