INTRODUCTION

The liver is one of the most important organs for the survival of living organisms. It performs a wide range of functions including detoxification, protein synthesis, and the production of numerous biochemics. Malfunction or dysfunction of hepatocytes leads, in many cases, to incurable liver diseases. Until now, orthotopic liver transplantation has been the only available treatment for patients with end-stage liver diseases. However, the use of this procedure is limited by the availability of donor organs. Hepato-

Possibility of Undifferentiated Human Thigh Adipose Stem Cells Differentiating into Functional Hepatocytes

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Background This study aimed to investigate the possibility of isolating mesenchymal stem cells (MSCs) from human thigh adipose tissue and the ability of human thigh adipose stem cells (HTASCs) to differentiate into hepatocytes.

Methods The adipose-derived stem cells (ADSCs) were isolated from thigh adipose tissue. Growth factors, cytokines, and hormones were added to the collagen coated dishes to induce the undifferentiated HTASCs to differentiate into hepatocyte-like cells. To confirm the experimental results, the expression of hepatocyte-specific markers on undifferentiated and differentiated HTASCs was analyzed using reverse transcription polymerase chain reaction and immunocytochemical staining. Differentiation efficiency was evaluated using functional tests such as periodic acid schiff (PAS) staining and detection of the albumin secretion level using enzyme-linked immunosorbent assay (ELISA).

Results The majority of the undifferentiated HTASCs were changed into a more polygonal shape showing tight interactions between the cells. The differentiated HTASCs up-regulated mRNA of hepatocyte markers. Immunocytochemical analysis showed that they were intensely stained with anti-albumin antibody compared with undifferentiated HTASCs. PAS staining showed that HTASCs submitted to the hepatocyte differentiation protocol were able to more specifically store glycogen than undifferentiated HTASCs, displaying a purple color in the cytoplasm of the differentiated HTASCs. ELISA analyses showed that differentiated HTASCs could secrete albumin, which is one of the hepatocyte markers.

Conclusions MSCs were isolated from human thigh adipose tissue differentiate to hepatocytes. The source of ADSCs is not only abundant abdominal adipose tissue, but also thigh adipose tissue for cell therapy in liver regeneration and tissue regeneration.

Keywords Thigh / Adipose tissue / Stem cells / Hepatocytes / Cell Differentiation
cyte transplantation could be employed as an alternative therapeutic approach to whole liver transplantation for liver failure. Recent studies have suggested that stem cell-derived hepatocytes could be utilized as therapeutic hepatocytes [1-5]. Bone marrow-derived mesenchymal stem cells (BM-MSCs), which possess characteristics such as self-renewal, pluripotency, proliferation, longevity, and differentiation, could be one of the sources for transplantable and therapeutic cells [6,7]. However, traditional bone marrow procurement procedures may be distressing to patients and yield a low number of MSCs when performed. Therefore, many researchers have investigated alternative stem cell sources for replacing BM-MSCs.

Adipose tissue commonly obtained using liposuction during plastic surgery is an attractive source of human MSCs, because it is a safe and abundant tissue in the body compared with other tissues. The stem cells in adipose tissue that are capable of differentiating into functional hepatocyte-like cells have phenotypic characteristics similar to BM-MSCs [8,9]. Several studies have reported the differentiation of hepatocyte-like cells from human adipose tissue-derived mesenchymal stem cells [1-3]. Seo et al. [4] demonstrated for the first time that human adipose tissue-derived mesenchymal stem cells differentiate into hepatocyte-like cells upon treatment with a hepatocyte growth factor (HGF), oncostatin M (OSM), and dimethyl sulfoxide (DMSO). These cells expressed albumin (ALB), α-fetoprotein (AFP), and other hepatocyte-specific markers during hepatic differentiation and increased in low-density lipoprotein (LDL)-uptake and urea production. In most of the reports on adipose-derived stem cells (ADSCs), the source of the adipose tissue used has been abundant abdominal adipose tissue. However, thigh adipose tissue that is commonly obtained using liposuction in plastic surgery can also be used as a source of ADSCs.

In this study, MSCs were isolated from human thigh adipose tissue and characterized for morphology, growth potency, phenotypic profile, and the capacity of differentiating into hepatic lineages. In addition, the ability of human thigh adipose stem cells (HTASCs) to differentiate into hepatocytes was evaluated.

**METHODS**

**Hepatic differentiation protocol**

Passages of 3 to 7.5 × 10^3 cells were plated onto a collagen coated 48-well culture plate, and hepatic differentiation was then performed over a period of three weeks using a three-step protocol (Fig. 1). At step I for the initiation, the cells were cultured for 4 days in serum-deprived Dulbecco’s modified Eagle’s medium with high glucose (DMEM-HG) supplemented with 100 μM L-ascorbic acid 2-phosphate (Asc 2-P) and 1× insulin-transferrin-selenium (ITS) consisting of 10 mg/mL insulin, 0.55 μg/mL transferrin, 0.67 μg/mL sodium selenite, 50 ng/mL activin A, and 20 ng/mL fibroblast growth factor (FGF4). At step II for the induction, they were cultured for 7 days in DMEM with low glucose (LG) supplemented with 10% fetal bovine serum (FBS), 100 μM A2P, 1 μM dexamethasone, 10 mM nicotinamide, and 1×ITS consisting of 20 ng/mL HGF, 20 ng/mL FGF4, 20 ng/mL epidermal growth factor (EGF), and 20 ng/mL OSM. During step III for the maturation, the cells were treated with DMEM-LG supplemented with 10% FBS, 100 μM Asc 2-P, 20 ng/mL OSM, 1 μM dexamethasone, 1% DMSO, and 1×ITS for 7 days.

**Reverse transcription-polymerase chain reaction**

Total RNA was isolated from undifferentiated or differentiated HTASCs using RNAiso kit (Takara, Otsu, Japan). The amount of RNA was assessed by measuring the ratio of absorbance at 260 nm to that at 280 nm (> 1.8). Reverse transcription polymerase chain reaction (RT-PCR) was carried out using a thermal cy-

### Fig. 1. The steps of differentiation

The steps of human thigh adipose stem cells differentiation into hepatocytes. FGF4, fibroblast growth factor 4; DMEM-HG, Dulbecco’s modified Eagle’s medium with high glucose; FBS, fetal bovine serum; Asc 2-P, L-ascorbic acid 2-phosphate; ITS, insulin-transferrin-selenium; HGF, hepatocyte growth factor; EGF, epidermal growth factor; OSM, oncostatin M; DMSO, dimethyl sulfoxide.
cler (GeneAmp PCR system 2400, Perkin-Elmer, Boston, MA, USA). A total of 7.5 μg (1 μg/μL) RNA was reverse-transcribed using the following RT mixture: 25 mM MgCl₂, 10× PCR buffer, 2.5 mM dNTPs mixture, 0.5 mg/mL oligo (d)T₁₄, 40 U RNase inhibitor, and 20 U AMV-RT. The RT reaction was allowed to occur for 60 minutes at 42°C, and the resultant cDNA was subjected to PCR amplification, using upstream and downstream primers of hepatocyte-specific genes as shown in Table 1. PCR was performed using the following PCR mixture: 25 mM MgCl₂, 10× PCR buffer, 2.5 U Thermus aquaticus (Taq) polymerase, and 100 pM sense and antisense primers. Amplification was performed for 35 cycles. Annealing temperature was depending

### Table 1. Primers used for the RT-PCR analysis for the stem cells and hepatocyte-related genes

| Gene   | Primer sequence                  | Size (bp) | Temp (°C) | Accession number |
|--------|----------------------------------|-----------|-----------|------------------|
| SCF    | 5′-CCA TTG ATG TCA GCT GCA-3′    | 275       | 55        | M59964           |
|        | 5′-CTT CCA GTA TCA GCC TCT AA-3′ |           |           |                  |
| Oct4   | 5′-CGT GAA GCT GGA GAA GAA GCT G-3′ | 245       | 55        | AF268617         |
|        | 5′-CAA GGG CCG CAG TGG ACA CAT TTT C-3′ | 331       | 55        |                  |
| Nanog  | 5′-ATG GCT ATG TGT GCT ATG AGC AG-3′ | 321       | 56        | Z19554           |
|        | 5′-CCT TCA AGC TCT TCT AGT CC-3′ |           |           |                  |
| Vimentin| 5′-CTT CGT GAA GTA CCA GGA ACA ATG ATA-3′ | 269       | 60        |                  |
| CK18   | 5′-GAG ATG GAC GCT CCT AAC AG-3′ | 357       | 57        |                  |
|        | 5′-CAA GGT GCC TCT CAG ATT CC-3′ |           |           |                  |
| FGF-5  | 5′-GCT GTG TCT CAG GGG ATT GAA GGA-3′ | 383       | 55        |                  |
|        | 5′-TAT CCA AGA GCA AAC ATG AGT CTG T-3′ | 294       | 60        |                  |
| NCAM   | 5′-GAG GGG GAA GTA GCC GCG TGT AAT G-3′ | 315       | 55        |                  |
|        | 5′-ATA TTC TGC TCT GCC CGG ATG GTA G-3′ | 220       | 60        |                  |
| Pax6   | 5′-AGA TCA AGA GGA GCC TCA AA-3′ | 313       | 57        |                  |
|        | 5′-ATT TGG GTG GTA GAC ACT GG-3′ |           |           |                  |
| BMP4   | 5′-AGC CAT AGT TTG ATC CC-3′    | 383       | 60        |                  |
|        | 5′-TCA GGG GAT CGT CGT AGG TT-3′ |           |           |                  |
| BMP2   | 5′-TTG C5G CAG CTC AGC ATG TT-3′ | 315       | 55        |                  |
|        | 5′-TTG CCA GAG CAT CAA GAT G-3′ |           |           |                  |
| HNF4α  | 5′-CAG CAG GAA TAG GAA GAG TTG-3′ | 205       | 62        |                  |
|        | 5′-GCC TGT CCT TGT GGA GTA AG-3′ |           |           |                  |
| HLA-ABC| 5′-GTA TTT TCT CAC ATC CCG GCG-3′ | 394       | 70        |                  |
|        | 5′-GTC GSC GSC GCT CCA AGA AGA GAC-3′ | 445       | 49        |                  |
| HLA-DR | 5′-CGT ATG GGT CGC CAG TCA TG-3′ | 220       | 60        |                  |
|        | 5′-GAG TCA CAG TCT TCT ATG AA-3′ |           |           |                  |
|        | 5′-TGG GAA GAA GAA AAT TCA AA-3′ |           |           |                  |
| ALB    | 5′-TTT ACC TTT TAG CAA AGA AAA GGA-3′ | 315       | 61        |                  |
| CX32   | 5′-GCC GTG AAC CGG CAT TCT AC-3′ | 400       | 61        |                  |
|        | 5′-ACA ACA GCC GGA ACA CCA CG-3′ |           |           |                  |
| Trans-ferrin | 5′-GTC GGC TTT GTC AAC AG-3′ | 255       | 52        |                  |
|        | 5′-CTC CAT CCA AGC TGA TG-3′    | 565       | 52        |                  |
|        | 5′-GTA CCA TTT CTT GAA GGT GAT T-3′ | 745       | 59        |                  |
|        | 5′-TAC AGC ACC CAA ATT TCA TA-3′ |           |           |                  |
| CYP2B6 | 5′-GAAG AGA AAG AAG AGC AG-3′   | 210       | 62        |                  |
|        | 5′-CAG CAA ACA CCT CTG CTA TTA-3′ |           |           |                  |
| CAR    | 5′-AAG GCC TCG TGA TCG AGT AA-3′ | 146       | 59        |                  |
|        | 5′-GAG GAT GAG CTC AGG AAG-3′  |           |           |                  |
| CK18   | 5′-GAG ATG AGA GCT CCT AAC AG-3′ | 265       | 57        |                  |
|        | 5′-CAA GCT GGC TCT CAG ATT TC-3′ |           |           |                  |
| HNF1α  | 5′-CAG TCT TCT TCT TCT GGA CTA GA-3′ | 357       | 57        |                  |
| HNF4α  | 5′-CTG GAG TTT GGA GAA GAA TGA-3′ | 321       | 60        |                  |
| GAPDH  | 5′-ACAACT TTG GTA TCG TG-3′     | 218       | 60        |                  |
|        | 5′-AAA TTC GGT TCT GTC ATA CCA G-3′ | 456       | 53        |                  |

RT-PCR, reverse transcription-polymerase chain reaction.
on the species of primers. The PCR products were mixed with 6× loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 40% sucrose) and separated on 2% agarose gels. After electrophoresis, the gels were stained with ethidium bromide. DNA signals on the gel were imaged under ultraviolet light using a Bioprofile Image Analysis System (UV Viewing Cabinet, Vilber Lourmat, Marne la Vallee, France).

**Immunocytochemical analysis**

The immunophenotype of the cells was determined using a stain kit (Vector Laboratory, Burlington, CA, USA) according to the manufacturer’s instructions. After the cells were cultured onto 8-well culture dishes, they were washed with Dulbecco’s phosphate buffered saline (DPBS) three times and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 hour at 4°C. They were washed with PBS and then permeabilized with 0.5% Triton X-100 in DPBS for 10 minutes at room temperature. After several washings, the cells were incubated in 3% hydrogen peroxide for 15 minutes to quench the endogenous peroxidase activities. They were then rinsed with DPBS and incubated in a blocking solution containing 2.5% horse serum for 30 minutes at room temperature with gentle shaking. The cells were incubated with mouse monoclonal antibodies against human albumin (ALB, 1:100), which had been diluted in 0.5% horse serum. After incubation with the primary antibodies for 17 hours at 4°C with gentle shaking, the cells were washed and rinsed with DPBS. They were incubated with a biotinylated universal antibody horse anti-rabbit/mouse IgG for 30 minutes at room temperature. The cells were rinsed several times, and then incubated with avidin-biotin complex (ABC) reagent conjugated streptavidin for 10 minutes at room temperature. Immunoreactivity was visualized utilizing 3,3′-diaminobenzidine tetrahydrochloride (DAB). The color reaction was stopped with water. Finally, they were counterstained with Mayer’s hematoxylin for 1 minute, rinsed in H2O, and observed for the purple staining indicative of the presence of glycogen using a light microscope.

**Statistical analysis**

Statistical analyses of this data were performed using the t-test. P-values < 0.05 were considered statistically significant. All results were derived from at least three independent experiments.

**RESULTS**

**In vitro differentiation of HTASCs into hepatocyte-like cells**

Growth factors, cytokines, and hormones were added to the collagen coated dishes to induce the undifferentiated HTASCs to differentiate into hepatocyte-like cells. During step I for initiation, cell morphology was not greatly changed by the treatment of activin A and FGF4 in the serum-deprived media. After 18 days of culture in hepatocyte differentiation media, the cells were transferred to starvation media deprived of serum, and the supernatant was harvested 20 hours later. The human albumin concentration in the supernatant was determined using the Human Albumin ELISA Quantitation Kit (Exocell Inc., Philadelphia, PA, USA) according to the manufacturer’s instructions. Serial dilution of a stock solution of albumin was used to create a standard curve. Samples were analyzed in duplicate under each condition.
cells were cultured in the step II media containing HGF, OSM, and dexamethasone for 7 days, the cells gradually changed from a fibroblast-like morphology to a round or polygonal shape. Finally, the cells were cultured in the step III media including OSM, dexamethasone, and DMSO for more than 7 days for maturation; the majority of the cells changed into a more polygonal shape showing tight interactions between the cells (Fig. 2).

**Gene expression patterns of HTASC-derived hepatocyte-like cells**

To confirm the above results, the expression of hepatocyte-specific markers on undifferentiated and differentiated HTASCs was analyzed using RT-PCR and immunocytochemical staining. Undifferentiated cells expressed mRNA for CK18, HNF1α, and HNF4α, but not ALB, CX32, transferrin, UGT1A1, CYP2B6, or CAR. In contrast, differentiated HTASCs expressed or up-regulated mRNA for ALB, CX32, transferrin, UGT1A1, CYP2B6, CAR, CK18, HNF1α, and HNF4α (Fig. 3). Immunocytochemical analysis showed that they were intensely stained with an anti-albumin antibody compared with undifferentiated HTASCs (Fig. 4).

**Functional characterization of HTASC-derived hepatocyte-like cells**

Differentiation efficiency was also evaluated using functional tests such as PAS staining and detecting of albumin secretion level using ELISA. PAS staining showed that HTASCs submitted to the hepatocyte differentiation protocol were able to more specifically store glycogen compared with undifferentiated HTASCs, which displayed a purple color in the cytoplasm of differentiated HTASCs (Fig. 5). ELISA analyses showed that differentiated HTASCs had a significantly higher secreted albumin (0.15 ±0.057 μg/mL) than undifferentiated HTASCs (0.05 ±0.021 μg/mL, P <0.05) (Fig. 6), suggesting that differentiated HTASCs could secrete albumin, which is one of the hepatocyte markers.

**DISCUSSION**

Regenerative medicine involves the combination of cells and
scaffold materials that can be loaded with bioactive factors, ideally resulting in the regeneration or replacement of lost or damaged tissues and organs. Multiple cell sources have been investigated for their possible applicability in tissue engineering. Stem cells that characterize self-renewal and differentiation into multi-lineaged cells are being investigated for tissue engineering. Embryonic stem cells are the most potent stem cells. However, their use is controversial and has major ethical implications [10]. MSCs can be obtained from adults and are widely used because of their differentiation potential. In addition to bone marrow, periosteum [11] and muscle [12] also appear to be sources of MSCs. Particularly, adipose tissue that is commonly obtained using liposuction in plastic surgery is an attractive source of human MSCs because it is a safe and abundant tissue in the body compared with other tissues. In addition, the Korea Food and Drug Administration has granted permission to use self-adipose-derived stem cells for transplantation using a minimum process.

Notably, the fact that plastic surgeons can obtain abundant adipose tissue by liposuction makes the study of ADSCs very convenient. Therefore, many researchers use abundant abdominal adipose tissue as a stem cell source. However, although studies on hepatocyte differentiation using abdominal ADSCs have been reported, reports on hepatocyte differentiation using thigh ADSCs have not been documented. Therefore, ADSCs were isolated from human thigh adipose tissue in this study and their morphology, growth potency, phenotypic profile, and capacity for differentiating into various lineages were characterized. In addition, the ability of HTASCs to differentiate into hepatocytes was evaluated.

To recapitulate liver development, a three-step protocol with sequential addition of growth factors (FGF4, EGF, and HGF), cytokines (activin A, OSM), hormones (dexamethasone and insulin), nicotinamide, and DMSO was used in this study [13, 14]. Activin signaling at a level threefold above baseline dictates that the mesendoderm will move toward the endodermal cell fate [15]. HGF was originally identified and cloned as a morphogenic activator for a wide variety of cells and is known to play an essential role in the development and regeneration of the liver [5]. The foregut endoderm is induced to the hepatocyte lineage by the FGF family produced by the adjacent cardiac mesoderm, which are required to induce a hepatic fate and not the default pancreatic fate [16]. It has been reported that the treatment of cells with OSM, a member of the interleukin-6 cytokine family, increased the cell size of hepatocytes and enhanced cell differentiation and formation of bile canaliculi [17]. Although OSM alone had weak effects on hepatocyte functions, albumin secretion and CYP450 activity were greatly enhanced when combined with nicotinamide or DMSO [18]. DMSO has been known to maintain the functions of adult hepatocytes in vitro [13]. It has been reported that nicotinamide and DMSO remarkably enhanced the emergence of small hepatocytes, and that OSM also synergistically enhanced the selective growth of small hepatocytes and inhibited the growth of blood cell populations [18].

After differentiation, the cells were observed for the morphological changes, gene expression pattern, and functional activities of hepatocyte-like cells derived from HTASCs. Similar to other studies using ADSCs from abdominal adipose tissue [1, 4], these cells displayed a hepatocyte-like morphology such as a polygonal shape and cytoplasmic granulation with a more prominent nucleus. The cells also expressed hepatocyte-specific genes including ALB, CX32, transferrin, UGT1A1, CYP2B6, CAR, CK18, HNF1α, and HNF4α, which was consistent with the gene expression pattern from abdominal ADSCs in the report by Seo et al. [4]. Furthermore, these cells exhibited hepatocyte functions such as storage of glycogen, and expression and secretion of albumin, a major protein produced by the hepatocytes. Schmelzer et al. [19] examined the gene expression profile of hepatic cells ranging from the stem cell stage to the mature functional stage. They showed that hepatic stem cells expressed high levels of CK19, low levels of ALB, and no AFP or adult liver-specific proteins. In contrast, hepatoblasts expressed low levels of CK19, elevated levels of ALB, high levels of AFP, and low levels of adult liver-specific proteins.

Taken together, this study successfully isolated human thigh ADSCs, differentiated them into endodermal hepatocytes, and confirmed that the hepatocytes were functional. Therefore, the source of ADSCs is not only abundant abdominal adipose tis-
sue, but also thigh adipose tissue for cell therapy in liver regeneration and tissue regeneration.

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Stem cells are capable of self-renewal and multipotent differentiation into cells that comprise the various organs of the human body beginning in the embryonic period. Stem cells also play a critical role in regeneration of organ or tissue functions throughout the lifetime. Basically, there are two types of stem cells: one is embryonic stem cells derived from blastocytes, and the other is adult stem cells obtained from fully developed adult tissue or placenta. Embryonic stem cells proliferate easily due to excellent self-renewal potency in their undifferentiated state. However, doing so raises ethical questions. Therefore, research on adult stem cells is actively in progress for a new approach to treating as yet unconquered diseases.

Marrow stem cells as a representative of adult stem cells have been used in studies of differentiation into various tissues [1,2]. However, harvesting bone marrow is painful to patients and requires multiple samplings to obtain sufficient amounts for clinical use. Therefore, many researchers have begun to seek alternative sources of stem cells. Zuk et al. [3] reported multilineage cells in the extract of adipose tissue that they assumed to be stem cells, and the researchers named them “processed liposapirate.” Recently, these cells have come to be called adipose tissue-derived stromal cells or adipose tissue-derived stem cells [4,5]. These cells can be extracted and separated from adipose tissue, show stable growth and proliferation in a culture environment, and are able to differentiate to various cells like marrow stem cells [6].

Unlike most of studies of adipose tissue-derived stem cells, which have used abdominal fat, the authors used fat from the thigh and differentiated it to morphologically hepatocyte-like cells that showed albumin production and glycogen storage ability, which are distinct characteristics of functional hepatocytes. This finding may provide a foundation for a new method of treatment of hepatic failure.

Recently, differentiation of mesenchymal stem cells has been reported to differ according to its source tissue or the age of the donor [7]. However, the idea that the location of the fat harvest, sex, or age of the donor can influence differentiation characteristics is not yet supported by reliable data. In the authors’ paper, human thigh adipose stem cells were able to differentiate into hepatocytes like abdominal adipose tissue-derived stem cells or bone marrow stem cells, but understanding the clear distinction of hepatic differentiation between human thigh adipose stem cells and abdominal adipose tissue-derived stem cells or bone marrow stem cells, and direct human application of these adipose tissue-derived hepatic lineage cells requires further investigation.

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