



# Low-serum culture system improves the adipogenic ability of visceral adipose tissue-derived stromal cells

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## Abstract

In obese adipose tissue, infiltrating macrophages release proinflammatory cytokines that trigger insulin resistance. An adipocyte-based platform from visceral fat would be useful to elucidate the pathology of adipose inflammation and to develop therapeutic drugs for insulin resistance. ADSCs (adipose tissue-derived mesenchymal stromal cells) expanded from subcutaneous fat are intensively studied as sources for regenerative medicine. However, the adipocyte culture system from visceral fat tissue has not been utilized yet. We aimed to establish the bioactive adipocyte platform using ADSCs from visceral fat pad. Stromal vascular fractions were processed from epididymal fat pads of Sprague–Dawley rats and three human omental fat pads, and the ADSCs were expanded using a low-serum culture method. The responses of ADSCs and ADSC-adipocytes (their adipogenic lineages) to pioglitazone, a therapeutic drug for diabetes, were evaluated by gene expression and ELISA. ADSCs ( $1 \times 10^8$ ) were expanded from 10 g of rat epididymal fat pads or human omental fat pads over five passages. Cell surface marker expressions revealed that visceral ADSCs were equivalent to mesenchymal stem cells. ADSC-adipocytes expanded in the low-serum culture system significantly showed higher expression of adipogenic markers [PPAR (peroxisome proliferator-activated receptor)  $\gamma$ , LPL (lipoprotein lipase) and FABP4 (fatty acid-binding protein 4)] and adipocytokines [adiponectin, resistin, leptin, PAI-1 (plasminogen-activator inhibitor 1) and IL (interleukin)-10] than those expanded in a high-serum culture system. Pioglitazone accelerated the adipogenic induction and increased adiponectin expression in human ADSCs by  $57.9 \pm 5.8$ -fold (mean  $\pm$  S.E.M.) relative to control cells ( $P < 0.001$ ). Both in rat and human ADSC-adipocytes, TNF- $\alpha$  significantly induced proinflammatory cytokines [MCP-1 (monocyte chemoattractant protein-1) and IL-6] and suppressed adiponectin expression, while pioglitazone antagonized these effects. The present findings suggest that visceral ADSC-adipocytes expanded in low-serum culture would be useful for adiposcience and pharmacological evaluations.

Keywords: adipose-derived stromal cell; adipogenesis; thiazolidinedione

## 1. Introduction

Excess visceral fat gives rise to a state of chronic low-grade inflammation that contributes to insulin resistance and type 2 diabetes (Shoelson et al., 2006). Macrophages infiltrating the visceral fat are prominent sources of proinflammatory cytokines such as TNF (tumour necrosis factor)- $\alpha$  and IL (interleukin)-6, which promote systemic inflammation by secreting inflammatory adipokines into the portal circulation that drains the visceral fat (Fontana et al., 2007). The link between macrophages and visceral adipocytes is an essential target for medication against insulin resistance. An *in vitro* system based on human cell lines would be ideal for progressing pharmaceutical investigations. However, the commercially available human visceral adipocyte cell lines have associated problems, including low propagation, low adipogenesis and variations

among individual lines. Therefore, most researchers currently use rodent cell lines (e.g. mouse 3T3-L1 preadipocytes) or SVFs (stromal vascular fractions) prepared from total adipose tissue, followed by hormonal differentiation programs (Schaffler and Buchler, 2007). Adipocytes from rodents and humans demonstrate species-specific differences, and mature adipocytes differentiated from SVFs cannot be expanded. Based on these limitations, we focused on pluripotent MSCs (mesenchymal stem cells) that can effectively serve as a source for human adipocyte tissue from the visceral fat pad.

MSCs from bone marrow or subcutaneous adipose tissue have been well explored in terms of regenerative medicine. Both types of MSCs have equal potential to differentiate into cells and tissues of mesodermal origin, such as adipocytes, cartilage, bone and skeletal muscle (Schaffler and Buchler, 2007). For regenerative therapies, ADSCs (adipose tissue-derived stromal cells) have advantages because of the easy access to subcutaneous adipose

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**Abbreviations:** ADSCs, adipose tissue-derived mesenchymal stromal cells; ADSC-adipocytes, ADSC adipose lineage cells; AM, adipogenic medium; DMEM, Dulbecco's modified Eagle's medium; FABP4, fatty acid-binding protein 4; FBS, fetal bovine serum; FGF, fibroblast growth factor; hADSCs, human ADSCs; IL, interleukin; LPL, lipoprotein lipase; PPAR, peroxisome proliferator-activated receptor; qRT, quantitative real-time reverse transcriptase; rADSCs, rat ADSCs; SVFs, stromal vascular fractions; TBP, TATA box-binding protein; TNF, tumour necrosis factor; TZD, thiazolidinedione.

tissue and simple isolation procedures. Recently, we developed a method for expanding hADSCs (human ADSCs) by reducing the amount of serum in culture (Iwashima et al., 2008; Kondo et al., 2009). We demonstrated that hADSCs expand efficiently in media containing 2% serum and FGF (fibroblast growth factor)-2. The low-serum-cultured hADSCs express cell surface markers similar to those on bone marrow-derived MSCs and can differentiate into mesenchymal lineage cells. Of interest, low-serum-cultured hADSCs showed higher biological activities. Specifically, they secreted more vascular endothelial growth factor and hepatocyte growth factor than hADSCs cultured with 20% serum. With the aim of expanding adipocytes from the visceral fat pad, we applied the low-serum culture system to SVFs from rat epididymal fat pads and human omental fat pads. We found that MSCs expanded rapidly and constantly in the low-serum culture system. We then characterized the cells and investigated their potential for use as a cell-based drug evaluation system. Here, we report our novel cell lines obtained from visceral fat pads using the low-serum culture system, which would be valuable for both adiposclerosis and drug development.

## 2. Materials and methods

### 2.1. Preparation of culture media

The basal culture medium for ADSCs was reported previously (Iwashima et al., 2008). Briefly, the basal medium was a 3:2 mixture of DMEM (Dulbecco's modified Eagle's medium) (Nissui Pharmaceutical Co. Ltd) and MCDB 201 medium (Sigma-Aldrich) supplemented with 1 mg/ml linoleic acid albumin (Sigma-Aldrich), a 1% volume of 100 × ITS supplement (Sigma-Aldrich), 0.1 mM ascorbic acid phosphate ester magnesium salt (Wako Pure Chemical Industries Ltd), 50 U/ml penicillin and 50 µg/ml streptomycin (Meiji Seika Ltd). FBS (fetal bovine serum) (ICN Biomedicals Inc.) at concentrations of 2 and 10%, and human FGF-2 (PeproTech Inc.) at 10 ng/ml was added to the basal medium.

### 2.2. Expansion of ADSCs from adipose tissue

Approximately 10 g of epididymal fat pads from Sprague-Dawley rats or human omental fat pads were washed with DMEM/F12 medium supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin and cut into 1-mm<sup>3</sup> pieces. The omental fat pads were obtained after receiving written informed consent from patients undergoing surgery. All the tissue samples were used with approval from and according to the guidelines of the Ethical Committee of Nagoya University Medical School (Approval number: 323.3). Adipose tissue was digested in 2.0 ml of Hanks' balanced salt solution containing 1 mg/ml collagenase type I (Worthington Biochemical Corporation) with reciprocal shaking for 1 h at 37°C. After removing undigested tissues by passing the samples through a nylon mesh with a pore size of 100 µm, the SVF was precipitated by centrifugation of the filtrate at 1200 rev./min for 5 min at room temperature. The SVF was washed three times

by resuspension in DMEM/F12 medium and further centrifugation, and the number of nucleated SVF cells was counted by staining with Turk's solution (Nacalai Tesque Inc.). SVF cells ( $1.0 \times 10^5$ ) were seeded in 25-cm<sup>2</sup> T-flasks (Nunc) coated with human fibronectin (Sigma-Aldrich) and cultured in 5 ml of media at 37°C under a humidified atmosphere of 5% CO<sub>2</sub>/95% air. After 24 h, non-attached cells and the medium were removed, and the culture was continued by replacement of fresh medium every other day.

### 2.3. Growth rate of ADSCs

Epididymal adipose tissue obtained from four Sprague-Dawley adult male rats at 10 weeks of age was used for this experiment. Four preparations of SVF cells ( $6.0 \times 10^5$ ) from the combined fat pad pool from the four rats were cultured in the following culture media: HS(+), culture medium containing 10% FBS plus 10 ng/ml human FGF-2; LS (+), culture medium containing 2% FBS plus 10 ng/ml human FGF-2; HS(-), culture medium containing 10% FBS only and LS(-), culture medium containing 2% FBS only. The growth kinetics was calculated at specified time points, and the cells were examined with phase-contrast microscopy after the sixth passage.

### 2.4. Isolation and analysis of ADSC clones

rADSCs (rat ADSCs) were expanded in three different culture systems, HS(+), HS(-) and LS(+), for five passages, and then plated at limiting confluence to result in isolated single cells. Cultures were maintained until well-defined colonies had formed. Single rADSC-derived colonies were harvested using sterile cloning discs and expanded in HS(+) in 96-well plates for 1 week until they reached 70–80% confluence. The expanded clones were cultured in adipogenic medium for 14 days. Adipogenic differentiation was induced as previously described (Pittenger et al., 1999), with minor modifications. Briefly, the cells were incubated in AM (adipogenic medium) comprising DMEM, 10% (v/v) FBS, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 0.1 mM indomethacin (Wako Pure Chemical Industries Ltd), 10 mg/ml insulin (Sigma-Aldrich) and 1 mM dexamethasone (Sigma-Aldrich). For experiments assessing the effects of pioglitazone, indomethacin was removed from AM. All the clones were analysed for adipogenic potential by Oil Red-O staining.

### 2.5. qRT (quantitative real-time reverse transcriptase)-PCR

Total RNA was isolated from cultured ADSCs and ADSC adipose lineage cells (referred to as ADSC-adipocytes hereafter) using a FastPure<sup>®</sup> RNA Kit (Takara Bio Inc.). Pools of cDNA were synthesized from 0.5–2 mg of total RNA using a RevaTraAce<sup>®</sup> Kit (Toyobo). Real time-PCR was performed using an Mx3000P Real-Time PCR System (Stratagene) using qPCR MasterMix Plus for SYBR<sup>®</sup> Green I Low ROX (Eurogentec). Specific primers were designed by an online service at the Universal Probe Library Assay Design Center (Roche Applied Science) in an intron-spanning manner for all possible cases. To avoid DNA contamination during

the RNA extraction process, every sample was treated with DNase I (Takara Bio Inc.). All primer pairs were confirmed not to self-dimerize by real-time PCR using a non-template control. Expression levels were calculated using relative standard curves for each mRNA of interest and housekeeping genes including 18S rRNA, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and TBP (TATA box-binding protein). For 18S rRNA and human TBP, TaqMan real-time PCR was performed using an 18S rRNA Control Kit and a human TBP Control Kit (both from Eurogentec), respectively. Further information is provided (Supplementary Table S1, at <http://www.cellbioint.org/cbi/035/cbi0350559add.htm>).

## 2.6. ELISA

Conditioned media were obtained from cultured ADSCs and adipocytes at 72 h after the final change of fresh DMEM containing 10% FBS. The adiponectin, MCP-1, IL (interleukin)-6 and TNF- $\alpha$  protein concentrations in the media were determined using ELISA kits (Quantikine Rat Adiponectin ELISA Kit, Quantikine Human Adiponectin ELISA kit, Quantikine Rat IL-6 ELISA Kit, Quantikine Human IL-6 ELISA Kit, Quantikine Rat MCP-1 ELISA Kit, Quantikine Human MCP-1 ELISA Kit, Quantikine rat TNF- $\alpha$  ELISA Kit; R&D Systems) according to the manufacturer's instructions.

## 2.7. Statistical analysis

Statistical analyses were performed using the software StatView 5.0 (SAS Institute). Two-way ANOVA (analysis of variance) was used to determine the significance of differences among the three groups. When a significant difference was indicated by ANOVA, further analyses were performed using the Bonferroni test to determine the differences between any pair of groups. A significant difference was defined as a value of  $P < 0.05$ . All data are provided as the mean  $\pm$  S.E.M.

# 3. Results

## 3.1. Expansion of ADSC from rat visceral fat tissue

Equal numbers ( $6.0 \times 10^5$ ) of SVF cells taken from rat epididymal fat pads were placed in the four different medium systems, containing 2 or 10% FBS with or without FGF-2. Rat ADSCs cultured in HS(+) grew the fastest, followed by those in LS(+) and HS(-) in the same range (Figure 1A). The cells cultured in LS(-) did not proliferate. The growth rate of subcutaneous fat pad rADSCs cultured in LS(+) was almost the same as that of visceral rADSCs (data not shown). Rat ADSCs grown in LS(+), HS(-) and HS(+) showed fibroblast-like morphology, but cell size was smaller in HS(+) than in LS(+) and HS(-) (Figure 1C). At earlier stages until the third passage, various forms of cells including epithelial-like cells that formed tubular structures and adipocyte-like cells containing lipid droplets were observed (Supplementary Figure S1 at <http://www.cellbioint.org/cbi/035/cbi0350559add.htm>). After four or five passages, these differentiated cells

disappeared, and monolayers of fibroblast-like cells were formed (Figure 1C). The expression of cell surface markers were monitored during the course of the expansion from the SVF to the fifth passage (Figure 1D, Supplementary Figure S2 at <http://www.cellbioint.org/cbi/035/cbi0350559add.htm>). The rADSCs from LS(+) and HS(+) constantly expressed positive markers for MSCs including CD10, CD90 and CD105. But in rADSC from HS(-), these markers gradually decreased after the second passages. The haematopoietic lineage markers including CD31, CD34, CD35 and CD45 that were negative for MSCs gradually reduced with every passage in all the culture systems. Expression of Pref1, a preadipocyte marker, was maintained in the same range in cells cultured in LS(+) and HS(+), but decreased in cells cultured in HS(-).

## 3.2. Single cell culture

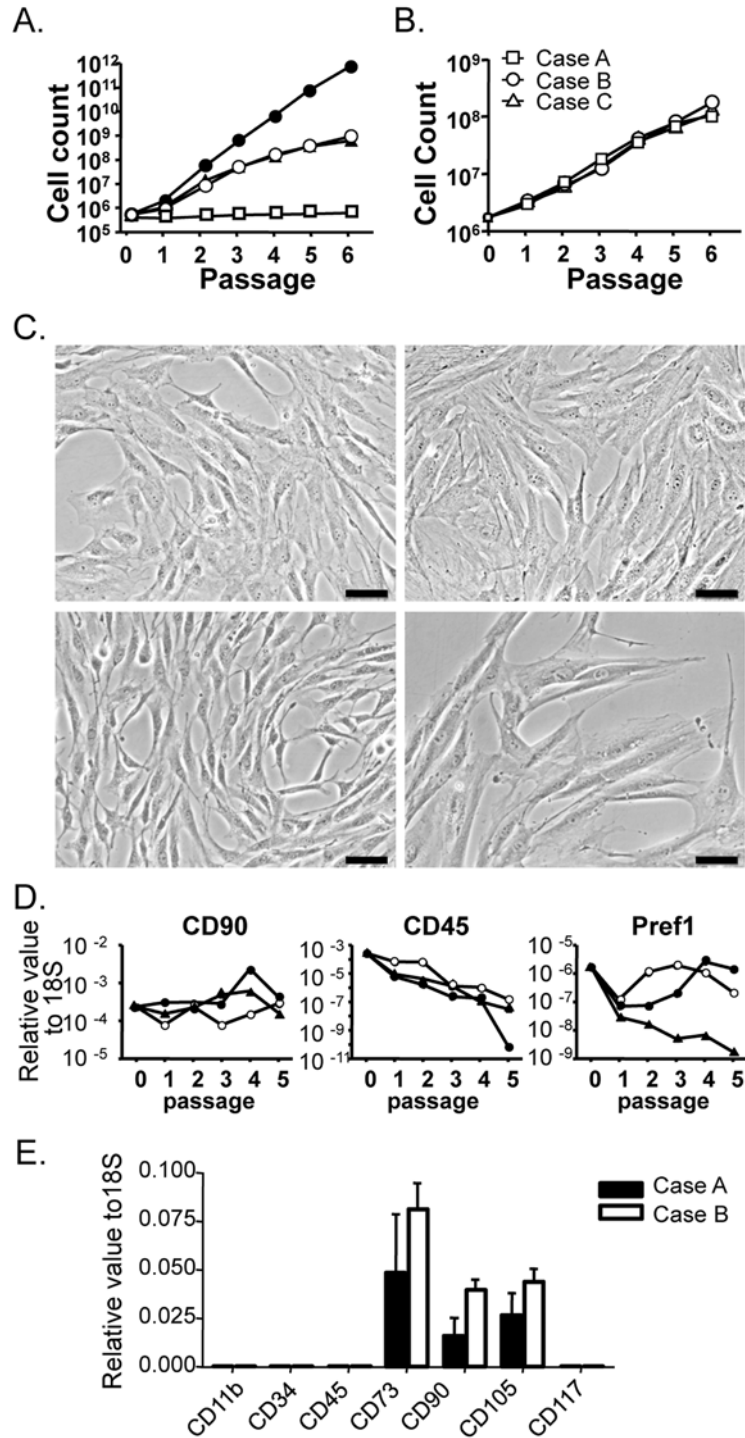
To select a suitable culture system for expanding stem cells with adipogenic ability, visceral rADSCs expanded in three different culture systems, LS(+), HS(+) and HS(-), were cultured at low confluence to allow the formation of single rADSC-derived colonies. More than 300 colonies were isolated and expanded for each culture system (Table 1). Adipogenic induction of rADSCs with AM resulted in an expanded cell morphology and a time-dependent increase in intracellular Oil Red-O staining (Supplementary Figure S3 at <http://www.cellbioint.org/cbi/035/cbi0350559add.htm>). The rate of adipogenesis was highest in LS(+)-cultured clones (77.5%), followed by HS(+)-cultured clones (58.9%) and HS(-)-cultured clones (22.2%).

## 3.3. Expansion of ADSC from human omental fat pads

Three lines of hADSCs were expanded in LS(+) from approximately 10 g of omental fat pads from three patients, whose profiles are shown (Supplementary Table S2, at <http://www.cellbioint.org/cbi/035/cbi0350559add.htm>). Each tissue was sampled during open surgeries for colon cancer, and no metastases to distal tissues including the omentum were recognized. The BMIs of the patients ranged from 19.6 to 25, and they did not have any metabolic diseases such as malnutrition or diabetes mellitus. After six passages in LS(+), each line of hADSCs from the three patients was expanded at almost the same rate, which exceeded  $1 \times 10^8$  cells (Figure 1B). The morphology of hADSC is fibroblast-like, quite the same as rADSCs, but the cell size is almost twice as large as rADSC (Figure 1C). There were no distinct differences in each hADSC from the three patients (data not shown). The expressions of cell surface markers in two lines of hADSC exhibited a similar pattern, which MSC-positive markers, including CD73, CD90 and CD105, were confirmed, and the cells did not express haematopoietic lineage markers, including CD11b, CD34, CD45 and CD117 (Figure 1E).

## 3.4. Expression of adipocyte-related genes during adipogenesis

rADSCs expanded in the three different culture systems were induced to undergo adipogenesis after the fifth passage when the homogeneous fibroblast-like cells reached 80–90% confluence.



**Figure 1** Expansion of ADSC from rat and human visceral fat pads

(A) Growth curve of ADSC from rat epididymal fat pads cultured under four different conditions: LS(-), open squares; LS(+), open circles; HS(-), closed triangles; HS(+), closed circles. (B) Growth curves of hADSC expanded in LS(+) from three cases. (C) Microscopic images of rADSCs. After six passages, either rat or human ADSCs change to homogenous spindle shapes. Upper left: rADSCs expanded in LS(+), upper right: rADSC in HS(-), lower left: rADSC in HS(+), lower right: hADSC in LS(+). Bars: 50 μm. (D) Expression of cell surface markers in rADSCs during expansion from the SVF to the fifth passage. The SVF from rat epididymal fat pads was subcultured using three culture systems: LS(+), open circles; HS(-), closed triangles; HS(+), closed circles. The cells were harvested after each passage and analysed by qRT-PCR to quantify the expression levels of surface markers. The relative expression of each gene with respect to the endogenous 18S rRNA is shown. The data points represent single assays. (E) Expression of cell surface markers in hADSCs after fifth passage. The data express mean value ± S.E.M. from triplicate assays.

**Table 1** Adipogenesis of single cell cultures of ADSCs raised in different *in vitro* culture systems

rADSCs were propagated in LS(+), HS(–) and HS(+) for five passages, followed by adipogenic induction for 21 days. At the end, each clone was stained with Oil Red-O, and the clones with accumulated lipid droplets were judged to be adipocytes.

Culture	LS(±)	HS(–)	HS(±)
Adipocyte	482	80	224
Other cell types	140	281	156
Total	622	361	380
Rate of adipogenesis (%)	77.5	22.2	58.9

Cells were maintained in AM for up to 22 days and harvested at specified time points. The expression of the mRNAs of interest were assessed by qRT-PCR (Figure 2A) or conventional RT-PCR (Supplementary Figure S4 at <http://www.cellbioint.org/cbi/035/cbi0350559add.htm>). Adipocyte-specific genes including PPAR ( $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ), LPL (lipoprotein lipase) and FABP4 (fatty acid-binding protein 4) were expressed along with adipogenesis in LS(+) and HS(+), but no changes were observed in HS(–). The lower expression of the pre-adipocyte marker, Pref-1, in the cells from LS(+) may suggest that most of the pre-adipocyte population has differentiated to mature adipocytes. The expression of adipocytokines associated with adipogenesis (adiponectin, leptin, resistin, PAI-1 and IL-10) were significantly increased. LS(+)-expanded cells expressed adipocyte-related genes at the highest levels. In contrast, the expressions of inflammatory cytokines (IL-6 and MCP-1) were decreased after the induction. The hADSC during adipogenesis also exhibited the same pattern of gene expressions as rADSC expanded in LS(+) (data not shown). Most of the mature ADSC-adipocytes contained multiple lipid droplets in their cytosol, but the cells with large single droplets (mono-ocular droplet) were fewer in hADSC-adipocytes (Figure 2BC). The PPAR $\gamma$  expression was significantly induced by adipogenesis in both rat and human ADSCs (Figure 2D), but the levels were higher in rADSC, which were 12-fold higher before the adipogenesis and 4-fold higher after the adipogenesis.

### 3.5. Pioglitazone promotes adipogenesis in ADSCs

To assess rADSC-adipocytes as a drug evaluation system, pioglitazone, a TZD (thiazolidinedione) compound used as a therapeutic drug for diabetes, was added to LS(+)-expanded rADSCs at 10  $\mu$ g/ml during adipogenic differentiation (Figure 3A). Indomethacin was removed from AM for these experiments to enhance the effect of the compound. Pioglitazone showed maximum effects at day 14 and increased the expression of the adipose tissue-specific transcription factor PPAR $\gamma$  by 150-fold before the induction, which was 6-fold higher than the pioglitazone (–) control ( $P < 0.001$ ). FABP4 and adiponectin expression were induced by approximately 20000-fold by pioglitazone, which were severalfold higher than the negative control ( $P < 0.001$ ). Pioglitazone suppressed TNF- $\alpha$  and PAI-1 expression at day 14, but did not change the IL-6 levels. The adipogenic activity of pioglitazone was also examined in the three lines of hADSCs (Figure 3B, Supplementary Figure S5 at <http://www.cellbioint.org/cbi/035/cbi0350559add.htm>). Pioglitazone significantly accelerated the adipogenic induction of PPAR $\gamma$  and adiponectin in each of the three lines. The accelerative effect on

PPAR $\gamma$  was significant, but limited, at  $1.49 \pm 0.27$ -fold higher than the pioglitazone (–) control (mean value of three lines  $\pm$  S.E.M.). In contrast, adiponectin and FABP4 were up-regulated by pioglitazone by as much as  $57.9 \pm 5.8$  and  $17.3 \pm 3.8$ -fold, respectively, compared with the control at day 21. The expressions of the inflammatory cytokines MCP-1 and IL-6 were markedly decreased after the adipogenic induction, and pioglitazone further suppressed these expressions compared with the control.

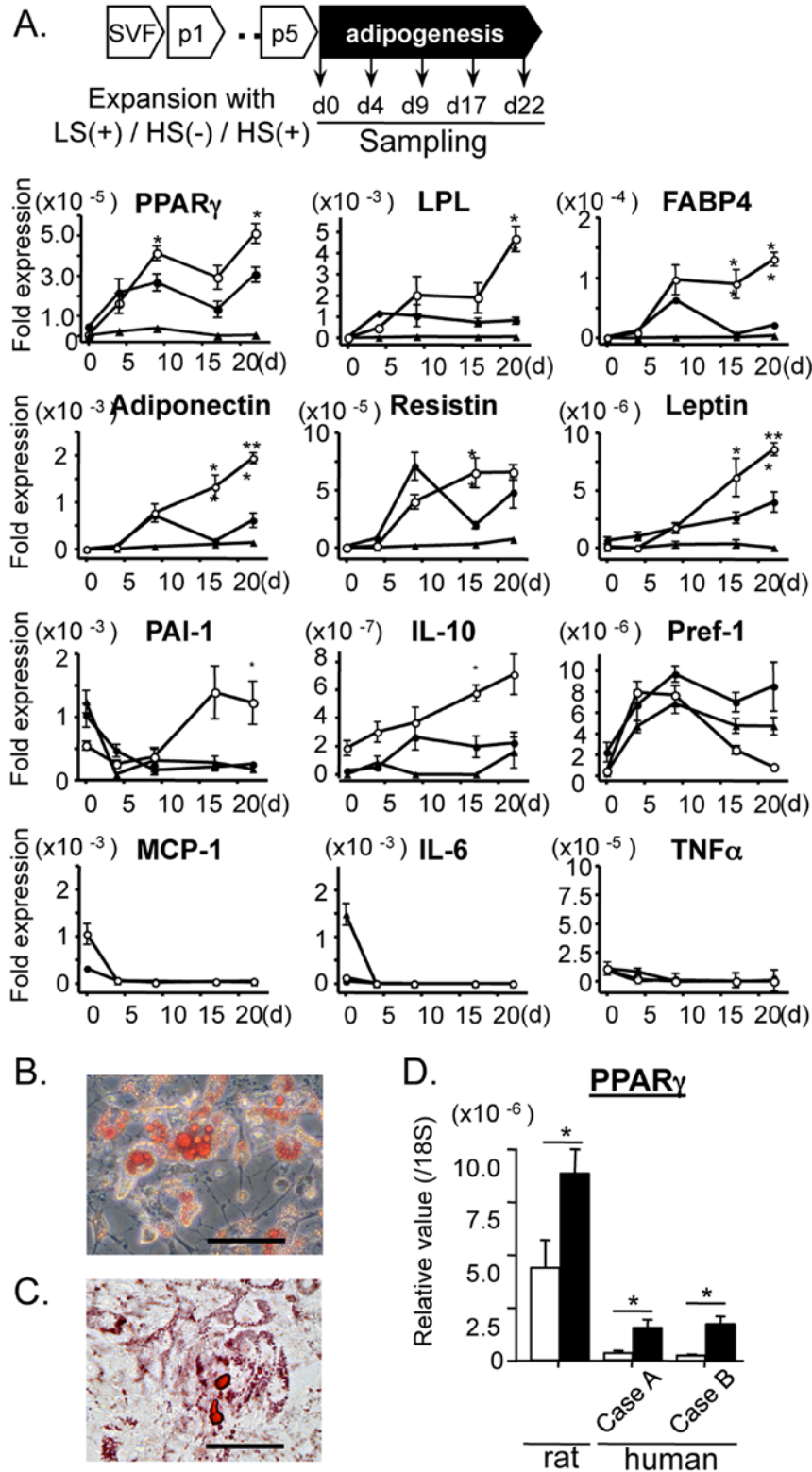
### 3.6. Pioglitazone antagonizes TNF- $\alpha$ in ADSC-adipocytes

TNF- $\alpha$  is a major macrophage-produced cytokine that is involved in chronic inflammation and is largely responsible for inducing insulin resistance in obese adipose tissue through its antagonism of the TZD/PPAR $\gamma$  system (Trujillo and Scherer, 2006). In mature rADSC-adipocytes, TNF- $\alpha$  induced the expression of inflammatory cytokines, including MCP-1, IL-6, and TNF- $\alpha$  itself, while suppressing the expression of adiponectin and PPAR $\gamma$  (Figure 4A). Pioglitazone effectively reversed the effects of TNF- $\alpha$  on MCP-1, IL-6, and TNF- $\alpha$ , adiponectin, and FABP4. The adipocytokine concentrations in the conditioned media measured by ELISA confirmed these changes in MCP-1, IL-6 and adiponectin (Figure 4B). In mature hADSC-adipocytes, TNF- $\alpha$  suppressed the expression of adipogenesis-related genes including PPAR $\gamma$ , FABP4 and adiponectin and up-regulated the expression of cytokines, including MCP-1 and IL-6, by up to 4-fold compared with the control (Figure 4C). For FABP4 and adiponectin expressions, pioglitazone antagonized TNF- $\alpha$  significantly but at limited levels. Pioglitazone did not significantly change these proinflammatory gene expression, although partial antagonism towards TNF- $\alpha$  was recognized for MCP-1 secretion (Figure 4D).

## 4. Discussion

In this study, we successfully established ADSC cell lines from visceral fat pads using a low-serum culture system, and showed that their adipogenic lineages are compatible with adipocyte cell lines. Our novel protocol using less serum and FGF-2 is suitable for use as *in vitro* expansion system for visceral adipocytes and is potentially useful for drug evaluation and adiposcience.

MSCs are considered to be multipotent cells present in the adult marrow that can replicate as undifferentiated cells and have the potential to differentiate into lineages of mesenchymal tissues, including fat, bone, cartilage, tendon, muscle and marrow stroma (Pittenger et al., 1999). Originally, MSCs were derived from



**Figure 2 Adipogenesis of rat and human ADSCs**

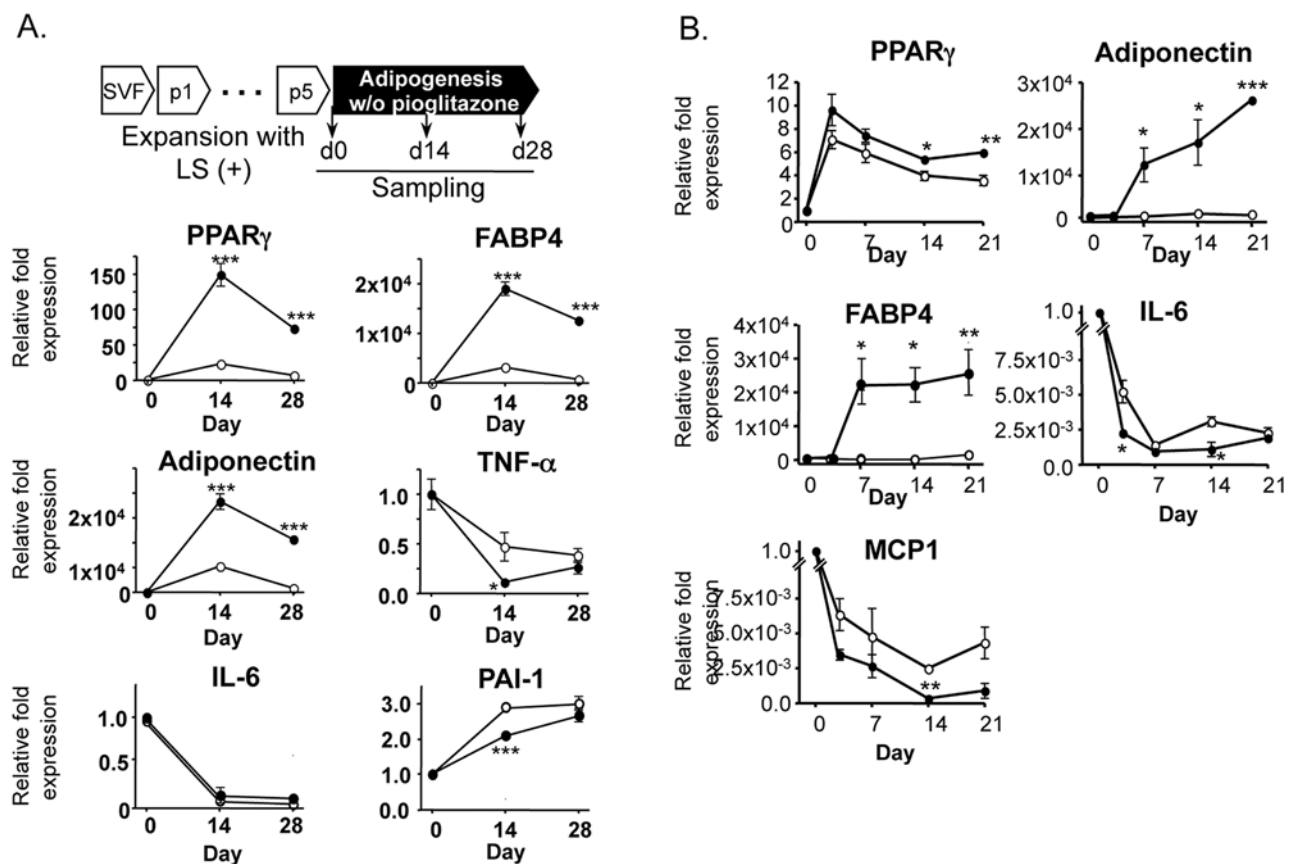
(A) Adipocytokine expression in rADSCs during adipogenic differentiation. ADSCs from rat epididymal fat pads were expanded in each of LS(+) (open circles), HS(-) (closed triangles) and HS(+) (closed circles) up to five passages, followed by adipogenic induction in adipogenic medium for up to 22 days. Quadruplicate samples of ADSCs were analysed by qRT-PCR to evaluate the expression of the indicated genes. The expression levels of each gene were normalized with respect to endogenous 18S rRNA expression, and the relative values to the gene expression at the onset of induction are shown. The data points represent the means  $\pm$  S.E.M. of four samples. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , significant difference compared with HS(+). Oil Red-O staining of

ADSC from rat (B) or human (C) after the adipogenic induction of 21 days. Bars: 100  $\mu$ m. (D) PPAR $\gamma$  expression in rat and human ADSCs before (open bars) and after (closed bars) 14 days of adipogenic induction. Cells were examined for PPAR $\gamma$  expression by qRT-PCR, and the relative expressions to 18S rRNA are shown as the means  $\pm$  S.E.M. of four assays.

plastic-adherent bone marrow cells. However, more recent studies have identified a number of other sources that contain MSCs at varying frequencies and with various differentiation capacities. These sources include adipose tissue, peripheral blood, umbilical cord blood, fetal hepatic and pulmonary tissues and placental tissue (Erices et al., 2000; Mareschi et al., 2001; Zvaifler et al., 2000). The pluripotent nature of MSCs has been attractive from the viewpoint of regenerative therapies. In addition, the cytokines produced by MSCs, which include vascular endothelial growth factor, hepatocyte growth factor and SDF-1, have proven beneficial for ischaemic diseases in various tissues.

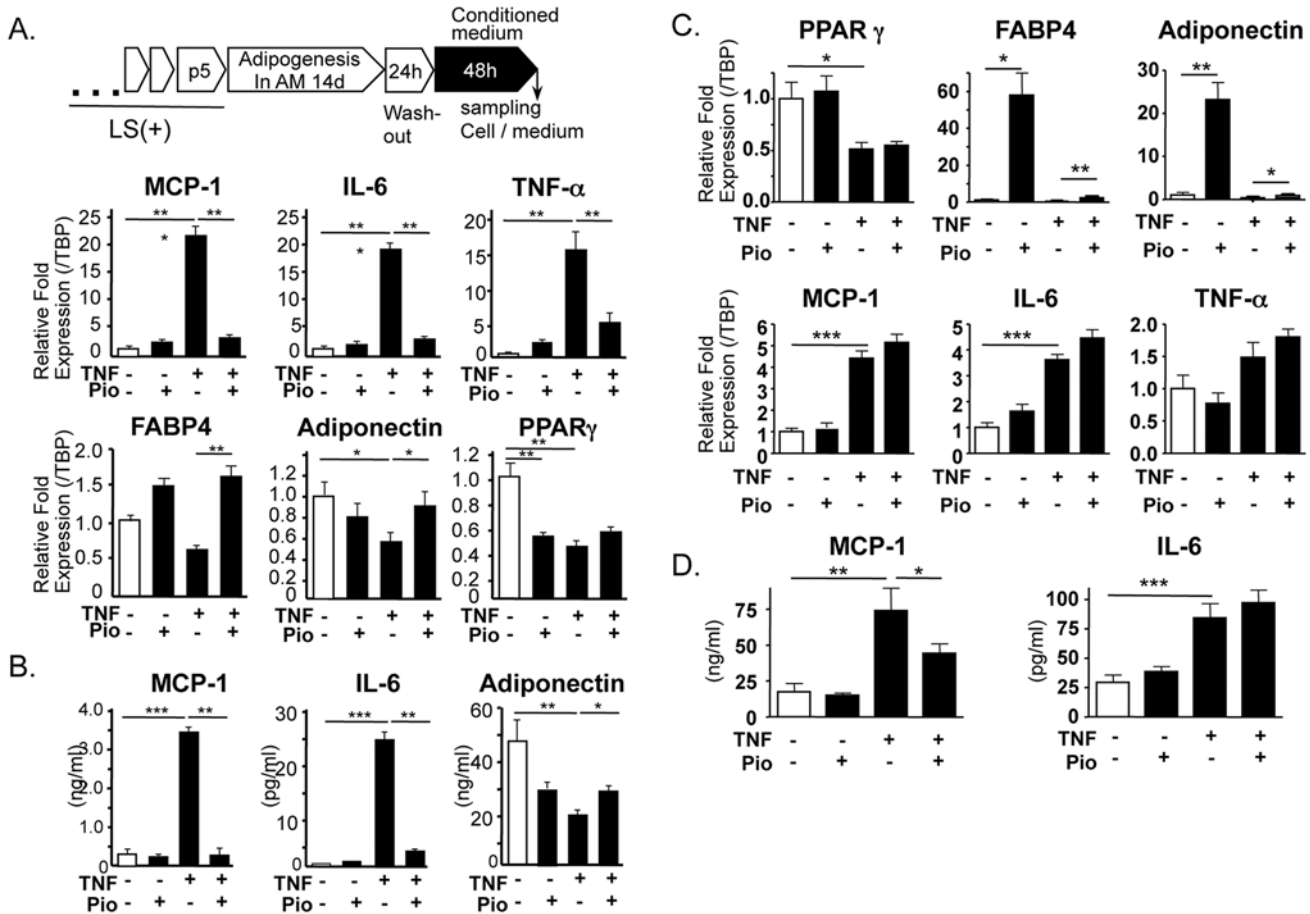
Recently, we developed a low-serum culture method that allows efficient expansion of bioactive MSCs from human subcutaneous fat pads (Iwashima et al., 2008; Kondo et al., 2009). The only essential growth factor used in the method is human FGF-2, which maintains or enhances the multilineage differentiation potential of MSCs (Battula et al., 2007; Tsutsumi et al., 2001), increases the proliferation rate of MSCs (Sotiropoulou et al., 2006) and suppresses the cellular senescence of MSCs (Ito et al., 2007).

In the present study, we applied this method to the expansion of visceral ADSCs. To date, most ADSCs have been obtained from subcutaneous fat pads owing to the easy access to and



**Figure 3** Pioglitazone promotes adipogenesis in ADSCs

(A) ADSCs from rat epididymal fat pads were subcultured using the LS(+) system for up to five passages, followed by adipogenic induction in AM for up to 28 days with (closed circles) or without (open circles) pioglitazone. Cells were harvested for RNA preparations at 0, 14 and 28 days after induction. Samples were analysed by qRT-PCR to evaluate the expressions of the indicated genes. The expression levels of each gene were normalized with respect to endogenous TBP expression, and relative values to the levels at the onset of induction are shown. The data points represent the means  $\pm$  S.E.M. of quadruplicate assays. Data were analysed by an unpaired *t* test. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001, significant difference compared with the respective pioglitazone(-) data. (B) hADSCs from one patient were subcultured using the LS(+) system for up to five passages, followed by adipogenic induction in AM for up to 21 days. The hADSCs were harvested for RNA preparations at 0, 3, 7, 14 and 21 days after adipogenic induction. Samples were analysed by qRT-PCR to evaluate the expression of the indicated genes. The expression levels of each gene were normalized with respect to endogenous TBP expression, and relative values to the levels at the onset of induction are shown. The data points represent the means  $\pm$  S.E.M. of quadruplicate assays. Data were analysed by an unpaired *t* test. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001 compared with the respective pioglitazone (-) data.



**Figure 4** Pioglitazone antagonizes TNF- $\alpha$  in the adipogenic lineage of ADSCs (A) Rat ADSC-adipocytes were incubated in conditioned media with or without TNF- $\alpha$ , and with or without pioglitazone for 48 h, followed by qRT-PCR (A) and ELISA (B) analyses. The data points represent the means  $\pm$  S.E.M. of sextuplicate assays. \*\* $P < 0.01$ , \*\*\* $P < 0.005$ . Human ADSC-adipocytes were incubated in conditioned media with or without TNF- $\alpha$ , and with or without pioglitazone for 48 h, followed by qRT-PCR (C) and ELISA (D) analyses. The data points represent means  $\pm$  S.E.M. from sextuplicate assays. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

abundance of the tissue. However, visceral adipose tissue is known to be morphologically and functionally different from subcutaneous adipose tissue (Wajchenberg, 2000). The expression profiles of the genes are depot-specific, especially for adipocytokines and insulin signalling (Lefebvre et al., 1998; Montague et al., 1998; Vidal, 2001; Vohl et al., 2004), which mediate part of the relationship between visceral obesity and the associated proinflammatory prothrombotic state. These depot-specific profiles are retained in adipocytes differentiated *in vitro* from precursor stromal cells that reside in the adipose tissue (Perrini et al., 2008). Therefore, it is logical to establish an *in vitro* system from visceral adipocyte progenitors that may be directly involved in the pathogenesis of diabetes. ADSCs were expanded from the epididymal fat pads of male Sprague–Dawley rats or human omental fat pads, both of which are recognized as visceral fat depots and have been extensively investigated. As observed for subcutaneous ADSCs, visceral ADSCs expanded exponentially in the low-serum culture system containing FGF-2 as the only growth factor. Visceral ADSCs expressed positive molecular markers for MSCs including CD10, CD90 and CD105 as well as

the preadipocyte marker Pref-1 at high levels throughout the passages. In contrast, haematopoietic markers including CD31, CD34 and CD45 gradually decreased, indicating that the low-serum culture system eliminates haematopoietic cells in the SVF, while MSCs survive. During the expansion from the SVF to the fifth passage, visceral ADSCs maintained their expression of the preadipocyte marker Pref-1. This suggests that a subpopulation of preadipocytes constantly exists at the same level during the culture. The adipogenesis of visceral ADSCs mirrored the gene expression patterns described for preadipocyte cell line adipogenesis, in which PPAR $\gamma$  expression was induced first, followed by expression of adipocyte markers including LPL, FABP4, adiponectin, resistin and leptin. The adipocyte markers were expressed at the highest levels in the ADSCs expanded in the low-serum culture system. These observations are reinforced by the results from single cell cultures, in which ADSC clones cultured in LS(+) contained the highest rate of adipocyte colonies. These findings indicate that the LS(+) culture system is the most suitable method in terms of preadipocyte expansion from visceral ADSCs.

With the aim of examining ADSC-adipocytes as a drug assessment platform that can substitute for visceral fat tissue, pioglitazone, a PPAR $\gamma$  agonist that is widely prescribed in the pharmaceutical market, was tested using the system. Pioglitazone accelerated adipogenesis and related gene expression in both rADSC-adipocytes and hADSC-adipocytes. Pioglitazone at 10  $\mu$ M up-regulated adiponectin expression by more than 50-fold relative to the control, comprising higher levels of induction than its agonistic activities in PPAR $\gamma$  promoter assays that caused 27-fold induction compared with the control (Fukuen et al., 2005). These observations demonstrate the sensitivity of the ADSC-adipocyte system for the evaluation of adipogenesis. On the other hand, pioglitazone did not increase adiponectin secretion by mature rADSC-adipocytes. This can be accounted for by the previous observation that TZDs only recruit adiponectin in non-differentiated or immature adipocytes (Feige et al., 2007).

It is postulated that TNF- $\alpha$  from infiltrating macrophages causes proinflammatory changes in adipose tissue (Trujillo et al., 2006). Addition of TNF- $\alpha$  to mature ADSC-adipocytes mirrored these phenomena, with increased expressions of MCP-1 and IL-6 and decreased expression of adiponectin. Pioglitazone antagonized TNF- $\alpha$  in rADSC-adipocytes, as reported in 3T3-L1 adipocytes (Szalkowski et al., 1995). In hADSC-adipocytes, TNF- $\alpha$  successfully induced proinflammatory changes. However, pioglitazone showed limited antagonism towards TNF- $\alpha$ . This may arise because of the relatively lower expression of PPAR $\gamma$  that is not sufficient to antagonize the proinflammatory responses induced by TNF- $\alpha$ . The morphological difference that hADSC-adipocytes did not develop large lipid droplets may be associated with the lower expression of PPAR $\gamma$ . The discrepancy may arise from the differences in the species and/or the portions of the fat pads that the cell lines originate from. Further studies are required to clarify the genetic background of ADSCs expanded from various portions of visceral fat pads that include omental, peritoneal and perirenal fat pads. The present findings suggest that hADSC systems from omental fat pads are useful for the evaluation of drugs that may (i) accelerate adipogenesis, (ii) accelerate inflammatory changes and (iii) antagonize TNF- $\alpha$  action on a pathway other than PPAR $\gamma$ . In addition, when more hADSC lines have been established, it may be possible to study personal differences in drug sensitivity, which may lead to tailor-made medication for individuals.

Recently, it was reported that most adipocytes descend from a pool of proliferating progenitors that are already committed, either in the prenatal period or within 2 postnatal days, and that these progenitors reside in the mural cell compartment of the adipose vasculature (Tang et al., 2008). It is unclear whether the mural cell compartment comprises the pluripotent MSCs themselves or committed preadipocytes. In either case, the SVF from adipose tissue includes a certain amount of adipocyte progenitors that can be efficiently expanded by the low-serum culture system.

The low-serum culture system provides more than  $1 \times 10^8$  ADSCs from 10 g of visceral fat tissue, which is the therapeutic target for diabetes. After adipogenic induction, ADSC-adipocytes provide an alternative *in vitro* system to human visceral fat pads that shows equal adipogenic capacity compared with mouse

3T3-L1 cell lines and is more stable than the primary culture system of mature adipocytes using the ceiling culture method.

#### Author contribution

Hiroshi Nagasaki was responsible for promoting the whole project, planning, sample collecting, gaining informed consent and performing all the experiments. QingLong Shang was responsible for the ADSC cultures, executing qPCR (quantitative real-time PCR) studies for Figures 3A, 4A and 4B. Takeshi Suzuki was responsible for setting up human and rat ADSC experiments for Figures 4A and 4B. Hiroyuki Hashimoto was responsible for setting up the qPCR studies for Figures 4A and 4B. Tomoko Yoshimura was responsible for the human ADSC cultures, executing qPCR studies for Figures 3B and S5. Taka-aki Kondo was responsible for the rat ADSC cultures for Figure S1. Takenori Ozaki and Shouichi Maruyama were responsible for setting up the ADSC cultures. Takahito Jomori designed the experiments for Figures 4A and 4B. Yutaka Oiso and Yoji Hamada were responsible for the general designs for the project and helped gain informed consent.

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